JOSIANE APARECIDA MARTINIANO DE PÁDUA

IDENTIFICATION OF IMMUNOGENIC EPITOPES FOR DESIGN VACCINES AGAINST *Leishmania* spp. IN DOGS: SISTEMATIC REVIEW

LAVRAS – MG

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Dissertation presented to the Universidade Federal de Lavras, as part of the requirements of the Programa de Pós-Graduação em Ciências Veterinárias, concentration area of Animal and Public Health, to obtain the Master's degree.

Dra. Ana Paula Peconick

Advisor

Dr. Ricardo Toshio Fujiwara

Co-advisor

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IDENTIFICAÇÃO DE EPITOPOS IMUNOGÊNICOS PARA VACINAS DE PROJETO CONTRA *Leishmania* SPP. EM CÃES: REVISÃO SISTEMATICA

Dissertation presented to the Universidade Federal de Lavras, as part of the requirements of the Programa de Pós-Graduação em Ciências Veterinárias, concentration area of Animal and Public Health, to obtain the Master's degree.

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Dra. Ana Paula Peconick	Universidade Federal de Lavras
Dr. Ricardo Toshio Fujiwara	Universidade Federal de Minas Gerais
Dr. Gabriel Andres Tafur-Gómez	Universidad de Ciencias Aplicadas y Ambientales

Lavras – MG

2022

Aos meus pais, por serem minha base e por me tornarem quem sou.

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"Entenda os seus medos,

mas jamais deixe que eles sufoquem os seus sonhos"

(Alice no país das maravilhas)

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RESUMO

A Leishmaniose Visceral é uma doença zoonótica, que atinge diversas espécies de mamíferos, dentre elas os cães, considerados os principais reservatórios no ambiente urbano. Para o controle e prevenção da doença em cães, a vacinação é considerada uma boa medida. Portanto, o objetivo do presente trabalho foi identificar epítopos protetores contra protozoários do gênero *Leishmania*, em cães domésticos (*Canis lupus familiaris)*, por meio de ferramentas de bioinformática, com pesquisas iniciais de antígenos em artigos científicos, agrupados em uma revisão sistemática e no banco de dados público TriTrypDB: *Kinetoplastid Genomics Resource,* com análises subsequentes de epítopos de células T e B no *Immune Epitope Database and Analysis Resource,* (IEDB), no qual a pesquisa foi limitada por meio de filtros de busca, com intuito de determinar as sequências genéticas dos peptídeos, que podem ser utilizados posteriormente na produção de uma vacina recombinante efetiva contra leishmaniose visceral canina. Como resultados principais vale destacar as taxas de eficácia significativas alcançadas por antígenos de segunda e terceira geração de vacinas. Os antígenos excretados/secretados de *L. infantum* e o antígeno LACK apresentaram proteção contra a Leishmaniose Visceral Canina consideráveis, tendo este último alcançado proteção também contra a infecção por *L. infatum.* No entanto, para a confecção de uma nova vacina multiepitopo, os LiESp não puderam ser considerados e por isso, considerou-se para a obtenção de dados no TriTrypDB e IEDB, apenas os antígenos LACK e A2, que apesar de não apresentar proteção significativa, mostrou relevância científica na literatura. É possível concluir que estes antígenos podem ser considerados para o desenvolvimento da nova vacina, por apresentarem relevância quanto às taxas de eficácia, entretanto, são necessárias maiores análises de seus epítopos para cumprir com este objetivo, pois para o desenvolvimento de um imunógeno a partir dessas moléculas, são necessários inferir características como sequência genética, comprimento, peso molecular, antigenicidade e diversas outras, com o intuito de selecionar os melhores epítopos e a melhor forma de fundi-los em um novo antígeno.

Palavras-chave: Bioinformática; Leishmaniose Canina; Vacina Recombinante

ABSTRACT

Visceral Leishmaniasis is a zoonotic disease that affects several species of mammals, including dogs, considered the main reservoirs in the urban environment. For the control and prevention of the disease in dogs, vaccination is considered a good measure. The objective of the present paper was to identify protective epitopes against protozoa of the genus *Leishmania*, in domestic dogs (*Canis lupus familiaris*), through bioinformatics tools, with initial antigen searches in scientific articles, grouped in a systematic review and the public database TriTrypDB: Kinetoplastid genomics Resource, with subsequent analyzes of T and B cell epitopes in Immune epitope Database and Analysis Resource, (IEDB), in which the search was limited using filters, to determine the genetic sequences of the peptides, which can be used later in the production of an effective recombinant vaccine against canine visceral leishmaniasis. As main results, it is worth noting the significant efficacy rates achieved by second and third-generation vaccines antigens. The excreted/secreted antigens of *L. infantum* and the LACK antigen showed considerable protection against Canine Visceral Leishmaniasis, the latter having also achieved protection against *L. infatum* infection. However, for the preparation of a new multiepitope vaccine, LiESp could not be considered and therefore, only the LACK and A2 antigens were classified to obtain data from the TriTrypDB and IEDB. A2 antigens, despite not showing significant protection, showed scientific relevance in the literature. It is possible to conclude that these antigens can be considered for the development of the new vaccine, as they are relevant in terms of efficacy rates, however, further analyses of their epitopes are necessary to fulfill this objective, because, for the development of an immunogen from these molecules, it is necessary to infer characteristics such as genetic sequence, length, molecular weight, antigenicity, and several others, to select the best epitopes and the best way to fuse them into a new antigen.

Keywords: Bioinformatics; Canine Leishmaniasis; Recombinant Vaccine

SUMMARY

1. PART ONE: THEORETICAL REFERENCE

1.1 Canine Visceral Leishmaniasis

Leishmaniasis is caused by obligate intracellular protozoa, belonging to the Phylum Sarcomastigophora, Order Kinetoplastida, Family Trypanosomatidae, and genus *Leishmania*, transmitted by insects of the subfamily Phlebotominae. (BAÑULS; HIDE; PRUGNOLLE, 2007). These parasites are classified as dimorphic, initially appearing in the promastigote form, which is elongated, flagellated, mobile, and found in the digestive tract of the insect vector (HANDMAN; BULLEN, 2002). This form, when encountering the cells of the mononuclear phagocytic system, undergoes several biochemical and metabolic changes and passes to the amastigote form, the non-flagellated stage of the parasite, which mainly infects phagocytic cells of the mammalian host and, when multiplying, can cause cell lysis, infecting others cells (PACE, 2014; SERAFIM; INIGUEZ; OLIVEIRA, 2019).

In the life cycle, the protozoan in its amastigote form, present in macrophages of vertebrate hosts, is ingested by the vector during the blood meal and goes to the posterior part of the insect's digestive tract (SERAFIM; INIGUEZ; OLIVEIRA, 2019). Under the influence of the pH of the medium, it is transformed into an immature form with no capacity for infection, called procyclic promastigote, which will multiply, mature, and migrate to the anterior part of the sandfly's digestive tract, being called metacyclic promastigote (BATES, 2018). This form of the parasite is not able to multiply, but it is capable to infect mammals in the next blood meal, being deposited on the host's skin (PACE, 2014). Thus, when it reaches the cells of the mononuclear phagocytic system, especially macrophages, they become amastigotes again, managing to multiply effectively and infect new cells and vectors (DA COSTA, et. al., 2019).

The protozoan is present in several parts of the world, and in 2018, ninety-two countries were considered endemic for visceral leishmaniasis and eighty-three were considered endemic for cutaneous leishmaniasis (WHO, 2022). Visceral leishmaniasis (VL), commonly caused by the species *L. donovani* and *L. infantum*, is considered the most severe form of the disease in humans (PACE, 2014). It is responsible for a clinical picture characterized by long periods of fever, fatigue, weight loss, hepatosplenomegaly, and lymph node enlargement, which can progress to more alarming signs and culminate in death (SAFAVI; ESHAGHI; HAJIHASSANI, 2020). Cutaneous leishmaniasis, characterized as the most common form of the disease, with 0.7 to 1.3 million cases per year worldwide, may present in a localized, diffuse, or mucocutaneous manner and is usually caused by the parasites of the species *L. major, L. amazonensis* and *L. braziliensis* (STEVERDING, 2017).

Of a zoonotic nature, the disease affects several species of mammals, with the canine species identified as the main reservoir of the parasite in the urban environment (GONÇALVES, et al., 2019). The participation of felines in the epidemiology of the disease is also highlighted, since feline leishmaniasis has been reported in several endemic countries, and can be pointed out as potential reservoirs of the protozoan (PENNISI; PERSICHETTI, 2018). In dogs, the illness presents in a severe form, clinically characterized by lymphadenopathy, skin lesions, onychogryphosis, drowsiness, anorexia, cachexia, conjunctivitis, polyphagia, epistaxis, locomotion problems, weight loss, and vomiting. Some may show little evident signs or no clinical signs at all (PICÓN, et al., 2020).

For the treatment in these animals, several drugs are mentioned, which are administered with the aim of reducing the parasite load, treating the damage caused, restoring the efficiency of the immune system, stabilizing the clinical picture, and preventing relapses (TRAVI, et al., 2018). Examples are meglumine antimoniate, with very satisfactory results for clinical cure in dogs, but without providing a parasitological cure, and allopurinol, used in long-term treatments, for helping to improve clinically and prevent relapses. It is also common to use a combination of the two drugs, bringing together the benefits of each one, without potentiating their toxic effects. (TORRES, et. al., 2011). There are also drugs such as aminosidine, amphotericin B, pentamidine, marbofloxacin, enrofloxacin, domperidone, among others, which can be used in different protocols according to the clinical stages of the disease, established in one of the many divisions such as A, B, C, D, Ea and Eb, ranging from dogs that do not need specific treatment for leishmaniasis to those that no longer respond to the recommended therapy (OLIVA, et. al., 2010). It is also necessary to highlight the active ingredient miltefosine, the only drug legally authorized as a therapeutic resource for dogs in Brazil, released through Technical Note N° 11/2016/CPV/DFIP/SDA/GM/MAPA, as it is not used in the treatment of human visceral leishmaniasis. This antiprotozoal, which also has antineoplastic activity, has good clinical results when used exclusively in treatment, being related to high rates of nephrotoxicity and hepatotoxicity (REGUERA, et al., 2016). Its efficiency can be increased when combined with other drugs, such as amphotericin B, which reduces the cost and time of treatment, improving the effectiveness of drugs in complicated cases (FÁLCI; PASQUALOTTO, 2015).

In terms of prophylaxis in canine species, it is necessary to use individual measures to protect dogs, such as collars impregnated with Deltamethrin 4% to reduce contact between animals and sandflies (SILVA, et. al., 2018), a measure regulated by the Ministry of Health of Brazil, for the control of visceral leishmaniasis in municipalities, through Technical Note No.

5/2021-CGZV/DEIDT/SVS/MS. It is also necessary to highlight the use of vaccines against the disease, however, so far, the existing vaccines are only partially effective against the various species of the *Leishmania* genus and therefore, the contribution of these products in relation to the control of the canine reservoir is not known (EVANS; KEDZIERSKI, 2012). In Brazil, there is only one commercially available vaccine registered with MAPA and it should only be administered to serologically negative dogs that do not show any clinical signs of the disease (CFMV, 2017).

As a measure to control the infected canine population, in Brazil, it is still recommended that animals be euthanized, however, due to problems such as lack of infrastructure in municipalities, lack of financial resources, and for various ethical reasons, it has been a method questioned as to its efficiency (COSTA, et al., 2020).

1.2 Relationship between host immune system, *Leishmania* spp. and vaccines.

One of the most important elements to determine the non-occurrence of clinical signs of leishmaniasis is the competence of the individual's immune system (FERNANDES, et al., 2012). Its capacity is influenced by several factors, such as coinfections, genetic factors, and nutritional deficiencies, as well as the interactions established between its components and the protozoan (MALAFAIA, 2009).

The immune system can be didactically divided into innate and adaptive. The innate immune system is composed of cellular components such as neutrophils, macrophages, natural killer cells, dendritic cells, eosinophils, basophils, and mast cells, in addition to diverse molecules such as those of the complement system, acute phase proteins, cytokines, and chemokines (CRUVINEL, et. al., 2010). It is characterized by providing protection to the individual without previous contact with the aggressor agent, reacting quickly in order to eliminate it from the body (MCCOMB, et al., 2019). The adaptive immune system is basically composed of T lymphocytes, B lymphocytes, antibodies, and other molecules, responsible for forming a more specific and specialized response against an agent, with the ability to generate immunological memory, from the previous contact with the microorganism (MCCOMB, et al., 2019). And it is on this principle of activating specific mechanisms of the immune system more quickly and effectively, from the previous contact with the microorganism, that vaccines are based, which are one of the most effective form of preventions and medical interventions against infectious diseases (POLLARD; BIJKER, 2021). They can activate the protection of the immune system, building a memory, without the individual suffering from the clinical signs or consequences of the disease (ZEEP, 2016).

Vaccines can be composed of live agents, which have gone through a process of attenuation and had their ability to cause the disease reduced or nullified (MINOR, 2015). Usually, they generate a strong response and lasting memory, by mimicking the infection caused by the pathogen in its wild form, having as main disadvantages the risk of pathogenicity reversion and the need for conservation under refrigeration (MINOR, 2015). They can also be composed of inactivated agents, in which the pathogen has been inactivated or killed by the action of chemicals or physical phenomena, maintaining the ability to stimulate the immune response and produce memory, of lesser intensity, but without risk of causing disease and therefore safe for immunocompromised people and pregnant women (KELLER-STANISLAWKI, et. al., 2014). Or even subunits, in which the molecules related to the pathogen are chosen according to their immunogenicity utilizing bioinformatics tools and can have their genetic sequences combined so that, at the end of the process, there is the formation of a specific immunogen (KALITA, et al., 2020). These immunogens have advantages about the cost of production, which on a large scale is relatively low, lower chances of adverse reactions and safer for immunocompromised and pregnant women, however, they usually need to be administered with stronger adjuvants, so that the induction of response be more efficient (NASCIMENTO; LEITE, 2012). And finally, composed of nucleic acids, using the agent's genetic material to produce immune response and memory (FRANCIS, 2018). When it comes to DNA vaccines, this genetic material can be coupled to a bacterial plasmid and have the advantage of a relatively simple and low-cost production, when compared to attenuated vaccines, for example, being stable at room temperature and being able to be used in different vehicles (LIU, 2011). On the other hand, RNA vaccines, approved for use in humans for the first time due to the SARS-CoV-2 pandemic, have the advantage of being able to be administered by different routes and, for better stability, they can be incorporated into different vehicles, such as lipid molecules and nanoparticles (BORAH, et al., 2021). They are based on the concept that, when this molecule reaches the interior of immune system cells, it can induce the production of target proteins directly in their cytoplasm, efficiently activating innate and adaptive immune responses (SANDBRINK; SHATTOCK, 2020).

Forms of artificial active immunization can be used as individual protective measures against Canine Visceral Leishmaniasis, which can directly influence the decrease in the population of canine reservoirs of the protozoan *Leishmania*, with a consequent drop in human cases of the disease (MARCONDES; DAY, 2019). For this to be achieved, the vaccine used must carefully selected antigens, adjuvants that help in the formation of an inflammatory response and be applied in the correct place and dose, so that they can stimulate innate and

adaptive mechanisms, which will help in the fight against this disease. to protozoa of the genus *Leishmania*. Among these mechanisms, the stimulation of IL-12 production by antigen-presenting cells (APCs) can be evidenced, with consequent activation of natural killer cells (LIU; UZONNA, 2012). In addition, there is also a decrease in the amount of IL-10 from T lymphocytes and APCs, with induction of a strong and long-term response, mediated by TCD4+Th1 and TCD8+ lymphocytes, responsible for the high concentrations of IL-2, IFN -γ and TNF-α (FERNANDES, et. al., 2012). All this will culminate in the high resistance of macrophages against *Leishmania* spp. infection, through the production of oxygen metabolites, such as superoxide anion, hydrogen peroxide and nitric oxide, responsible for eliminating the pathogen (TURCHETTI, et. al., 2015). In addition, there is also the formation of extracellular traps (NET) and secretion of enzymes by neutrophils, which, despite not being extremely efficient in the long term, manage to cause the death of the protozoan during the first days of infection (PEREIRA, et. al., 2017). There is also the production of antibodies by B lymphocytes, closely related to the clinical signs of the disease, due to the formation of immune complexes, which, when accumulating, can cause changes such as vasculitis, polyarthritis, skin ulcerations, uveitis and glomerulonephritis (CIARAMELLA; CORONA, 2003). These mechanisms are identified as part of a predominantly Th1-type immune response, a profile that stands out for being efficient in combating the protozoan (MORENO, 2019). Unlike the Th2 profile, which has cell types and cytokines such as IL-10, IL-4, IL-5 and IL-13, which create an environment related to the persistence of the parasite in individuals (JAIN; JAIN, 2015). In addition, the Th17 profile exerts regulatory functions of the immune system and may also be linked to the inability of the immune system to eliminate the parasite (KUMAR; NYLÉN, 2012). The predominance of these profiles is determined by genetic factors related to the host, the *Leishmania* species present in the individual, the number of parasites inoculated, as well as the inoculation site and salivary components of the insect vector (ROGERS, et. al., 2002). In this context, it is possible to determine criteria that make an animal resistant or susceptible and also define an ideal vaccine against Canine Visceral Leishmaniasis.

Among the various existing immunizing against the disease, the one composed by the Fucose and Mannose Ligand (FML) antigen, a surface antigen of the parasite, present throughout its life cycle and which proved to be a potent immunogen in BALB mice /C, Swiss albino mice and hamsters, in addition to being efficient in the treatment of dogs with subclinical infection (SANTOS, et al., 2003). This antigen, together with the adjuvant Saponin QuilA, was tested by Borja-Cabrera, et al. (2002) in 44 dogs domiciled in São Gonçalo do Amarante, in the state of Rio Grande do Norte, Brazil, compared to 41 unvaccinated dogs. In response, 100% of

vaccinated dogs demonstrated high antibody production after 2 years of vaccination. A protection rate of 95% was also found, indicated by the authors as also responsible for the decrease in human cases in the area during the study period. In addition to this, it is also necessary to highlight the immunogens composed of the recombinant antigens CPA and CPB, tested with IL-12 and IL-12 adjuvants plus QuilA in 5 dogs in each group, which did not show efficacy against the infection (POOT, et al., 2006) and DNA-LACK antigens with an additional dose of rVV – LACK, which showed 80% protection against the disease, when tested in 5 dogs (RAMIRO, et al., 2003). Many other vaccines have shown promising results, however, only three are vaccines approved for commercial use, two of them in Europe, Canileish ® (Virbac, France) and Letifend ® (Leti Laboratories, Spain), and one in Brazil, the Leish -Tec® (Hertape Calier, Brazil) (MORENO, 2019).

Letifend ® is composed of a chimeric protein called Protein Chimeric Q, which showed 72% protection against clinical signs in beagle dogs, when administered in two doses, without adjuvant, being highly immunogenic, stimulating a good humoral and cellular immune response (CARCELÉN, et al., 2009). The LiESP antigen added to the adjuvant QA-21, which makes up the Canileish ® vaccine, presented 68.4% of prevention of clinical signs of CVL, with 92.7% of protection, when tested by Oliva, et al. (2014), in Barcelona, Spain. The Leish -Tec® vaccine, the only one currently commercially available in Brazil, is composed of a recombinant form of the A2 antigen, derived from the *Leishmania* spp. to the saponin adjuvant, which satisfactorily stimulates the production of IFN-γ, with a decrease in IL-10 levels and an increase in the concentrations of IgG, IgG2, but not IgG1 antibodies, however, its effectiveness is 42.86%, the which demonstrates that the vaccine provides partial protection for animals (FERNANDES, et. al., 2008).

Considering the current limitations about vaccines against Canine Visceral Leishmaniasis, further studies on the subject are necessary, in an attempt to find a complete vaccine antigen that meets all the ideal characteristics and can protect against the disease and infection. Thus, the general objective of the present study was to identify immunogenic epitopes against protozoa of the genus *Leishmania*, in domestic dogs (*Canis lupus familiaris*), through research tools in scientific articles and bioinformatics.

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2. PART TWO: Article 1

This article was written according to the Instructions for authors of Transboundary and Emerging Diseases journal (Impact Factor: 5.005).

Efficacy of vaccines against Canine Visceral Leishmaniasis: A systematic review

Abstract

Canine Visceral Leishmaniasis is a zoonotic disease of great worldwide importance and can be prevented by vaccinating seronegative dogs. The objective of the present systematic review is to verify the effectiveness rate of vaccines, tested in dogs, against Canine Visceral Leishmaniasis (CVL) or *L. infantum* infection. This review was prepared using the PRISMA protocol, with the evaluation of studies obtained through searches in databases carried out by two independent reviewers and the resolution of divergences carried out by a third reviewer. The risk of bias analysis was performed using SYRCLE's RoB tool, resulting in the final analysis of 22 studies. 81.8% of the studies made experimental infection and 90.9% used individuals of both sexes. The vaccinated and control groups were predominantly composed of beagle animals, used in 63.64% of the studies. Among these, we highlight the article that evaluated the LiESP antigen added to the adjuvant QA-21, which showed 62% protection against CVL, the study that analyzed the LACK antigen, which showed 80% protection against CVL and 60% efficacy against *L. infantum* infection. Both studies were carried out with experimental challenge of their animals with selected strains of the protozoan. Another study, in which the immunogen LiESAp added to the adjuvant MDP, showed 100% protection against the disease, performed natural exposure of their dogs, which were vaccinated and submitted to an environment with the presence of the parasite and its vector. These antigens demonstrate significant effectiveness, being promising, after further studies, so that they can be commercially available as individual and collective prevention measures.

Keywords: Zoonosis, Leishmaniasis, Dogs, Efficacy, Protection

1- Introduction

Leishmaniasis is a complex of neglected diseases present in 98 countries in Europe, Africa, Asia and America, caused by obligate intracellular protozoa of the genus *Leishmania*, which infect an average of 0.9 to 1.7 million people every year (Steverding, 2017). In Central and South America, the most common species, called *L. infantum*, is commonly transmitted from animals to humans by the bite of the female sandfly *Lutzomyia longipalpis* (De Sousa-Paula et al., 2020). It is considered a public health problem, since the number of cases of the disease in dogs is directly related to the number of human cases of Leishmaniasis, because the parasite, residing in the skin of animals, is also transmitted vectorially to people (Marcondes & Day, 2019).

Dogs, considered the main reservoirs of the disease in an urban environment, may show clinical signs such as weight loss, lymphadenopathy, skin lesions, onychogryphosis, muscle atrophy and ocular signs (Moreno, 2019). It is also common the existence of resistant dogs, which do not show clinical signs, called asymptomatic and which, even so, are a source of infection for the sand fly (Shokri et al., 2017). The resistance of these dogs can be attributed to the actions of the immune system against the parasite. The Th1 immune response profile, in which there is stimulation of a response mediated by TCD4+Th1 and TCD8+ lymphocytes, is associated with a lower development of clinical signs in animals, with the participation of natural killer cells, with high production of IL-12, IL -2, IFN-γ and TNF-α and lower production of IL-10 (Fernandes et al., 2012). This environment is conducive to the classical activation of macrophages, which will produce oxygen and nitric oxide metabolites, responsible for destroying the protozoan inside the cell (De Vasconcelos et al., 2017). Contrary to what occurs in animals whose predominant profile is Th2, characterized by higher amounts of IL-4, IL-5, IL-10 and TGF-β and cells with a lower capacity to destroy the protozoan and, therefore, responsible for a greater susceptibility to infection and the appearance of clinical signs (Gonçalves et al., 2019).

These clinical signs can be treated with several drugs, including pentavalent antimoniates, which are drugs of choice for the treatment of CVL, however, they commonly cause gastrointestinal disorders, nephrotoxicity, skin irritation, hyperproteinemia and apathy (Reguera et al., 2016). The active ingredient miltefosine, in combination with allopurinol, is also commonly used, proving to be quite effective, as well as other drugs that, by reducing the

parasite load of infected dogs, reduce their infectivity, reducing epidemiological risks for humans and other animals (Travi et al., 2018), as well as the use of prevention methods.

CVL can be prevented by controlling the vector population, administering residual-effect insecticides in homes, applying protective screens on doors and windows, and cleaning environments conducive to the accumulation of organic matter (Gonçalves et al., 2019). Individual protection measures can also be applied, such as the use of 4% Deltamethrin repellent collars, preventing the movement of animals in environments where sandflies can inhabit, especially at twilight, and vaccination (Reguera et al., 2016). The vaccination is considered an individual protection measure because the vaccines available on the market protect against the disease, but not against infection in animals (Dantas-Torres, 2020). For this reason, it is necessary to develop a new vaccine antigen that protects against disease but also the infection, since decreasing the number of infected dogs, the probability of transmission of the parasite to the sand fly is also reduced and consequently the number of human cases of leishmaniasis. Therefore, it's necessary an antigen that stimulates immune mechanisms linked to animal resistance, in addition to meeting ideal characteristics such as a smaller number of applications and a lower cost. However, due to the complexity of the protozoan, this objective is still challenging, with only three vaccines available commercially in the world, which present partial protection against Canine Visceral Leishmaniasis (CVL) and do not represent a significant impact on the reduction of human cases of Leishmaniasis, since do not prevent infection in dogs (Dantas-Torres, 2020). Therefore, there are several studies aimed at the development of effective immunogens against CVL and the infection of animals by *L. infantum*, but many of them have not reached satisfactory levels of protection or need more adequate protocols to achieve these purposes. Therefore, the objective of this systematic review is to verify the effectiveness rate of vaccines against Canine Visceral Leishmaniasis or the parasite *L. infantum*, tested in dogs, to demonstrate which of them achieve the desired protection and which are the best protocols to be used.

2- Material and methods

The guidelines of PRISMA statement (Preferred Reported Items for Systematic Reviews and Meta-Analysis) were adopted in this review (Supplementary Table S1).

2.1- Strategy of search and selection of the studies

The review started with searches for studies in Pubmed, Web of Science, Scopus, Cochrane, Scielo and CABI databases on September 9, 2020 and the terms were searched by a

reviewer (JAMP) in the title, abstract and text sections complete. The PICOT (population, intervention, comparison, outcome and time) used for the searches, which involved the canine population, the different types of vaccines against Canine Visceral Leishmaniasis, used for prophylaxis, their efficacy and protection, is described in the Supplementary Table S2.

Initially, the studies found in the databases were added to a reference management software, where they were selected by title by two reviewers (JAMP and TFM), who also independently selected the studies by abstract. Titles that contained information about *Leishmania* species other than *L. infantum* or other host species other than the canine, were excluded, as was the case in the selection of studies by abstract. In a subsequent step, the full texts were analyzed by two reviewers (JAMP and TFM) and included or excluded based on predetermined criteria. Disagreements between the two reviewers were resolved by a third reviewer (EMSD).

2.2- Inclusion and exclusion criteria

The papers included in the review were those that fit the following criteria: (i) published in all countries, (ii) published in all years, (iii) that talked about vaccines used for prophylaxis, (iv) against Canine Visceral Leishmaniasis, (v) in dogs, (vi) *L. infantum* species. Studies in languages other than English, Spanish or Portuguese and which fit the exclusion criteria detailed in Supplementary Table S3 were excluded. This step was performed by two reviewers (JAMP and TFM).

2.3- Risk of bias analysis by SYRCLE's RoB Tool

After the inclusion of the papers by pre-established criteria, an internal validation was carried out by a specific protocol, created to analyze the risks of bias in studies with animals, called SYRCLE's RoB tool (Hooijmans, et al., 2014). This protocol consists of evaluating five different types of bias: (i) selection bias, (ii) performance bias, (iii) detection bias, (iv) attrition bias, (v) reporting bias and (vi) others biases. These five types of bias are divided into ten questions or domains that must be answered with “Yes”, “Unclear” or “No”, which respectively mean low, uncertain or high risks of bias.

2.4- Type of studies

Only original studies were included. Trials as cohort, case - control, cross sectional, case series, case reports and reviews were excluded.

2.5- Data extraction

Data were extracted by one of the reviewers (JAMP) and verified by two reviewers (MMO and EMSD). The author, the year of publication and the country where the study was carried out, and the type of vaccine antigen tested were first extracted. Subsequently, the characteristics of the evaluated groups were extracted, such as age, sex, total number of animals and per group, breed, dose, number of vaccinations, time between doses, type of adjuvant, and route of administration. As for the animals in the control group, the number of animals and what was applied to them, the period, dose and route of the experimental challenge and the period of exposure to the parasite were extracted. When it came to studies that performed natural challenge, the diagnostic methods used to confirm infection, as well as tests performed to verify the immunogenicity of the antigen, were the data extracted from each study for further analysis.

2.6- Statistical analysis

Statistical analyzes were performed using the GraphPad Prism Software (version 8.0). Chi-Square or Fisher's tests were applied exact for the calculation of Relative Risk (RR), considering a confidence interval of 95% ($p < 0.05$). To calculate vaccine efficacy, the formula 1-RR was used. Vaccine efficacy rates against *L. infantum* infection and the development of Canine Visceral Leishmaniasis, were calculated from data from animals, vaccinated and from the control group, considered positive in at least one diagnostic test among those performed by the study and from animals that presented characteristic clinical signs of the disease, respectively. Studies that did not assess infection by diagnostic methods or did not assess clinical signs were excluded from these analyses.

3- Results

3.1- Selected studies

In the initial search, 37.595 studies were found and, among them, 9.507 duplicates were detected by the reference management software, totaling 28.088 articles that were included in the initial selection by titles. The studies that contained the words “Canine” or “*Leishmania*” or “Vaccina” were kept for the selection of abstracts, also carried out using the same criteria. In the end, 76 papers were selected to be analyzed by quality criteria and 22 were eligible for the final analyzes (Table 1), which were submitted to risk of bias analysis by SYRCLE's RoB Tool (Figures 2 and 3). All these studies were carried out between 2003 and 2020. The 54 articles excluded after the selection stage by eligibility criteria, as well as the reasons why they were not included in this review, are shown in Supplementary Table S4. Studies that performed more

than one experiment, analyzed different antigens or the same antigen at different times, compared to more than one control group or with different challenge characteristics, such as time or dose, were defined as trials. In the end, 45 trials were included in this review.

Figure 1 – PRISMA flowchart used in the selection of the studies for this systematic review.

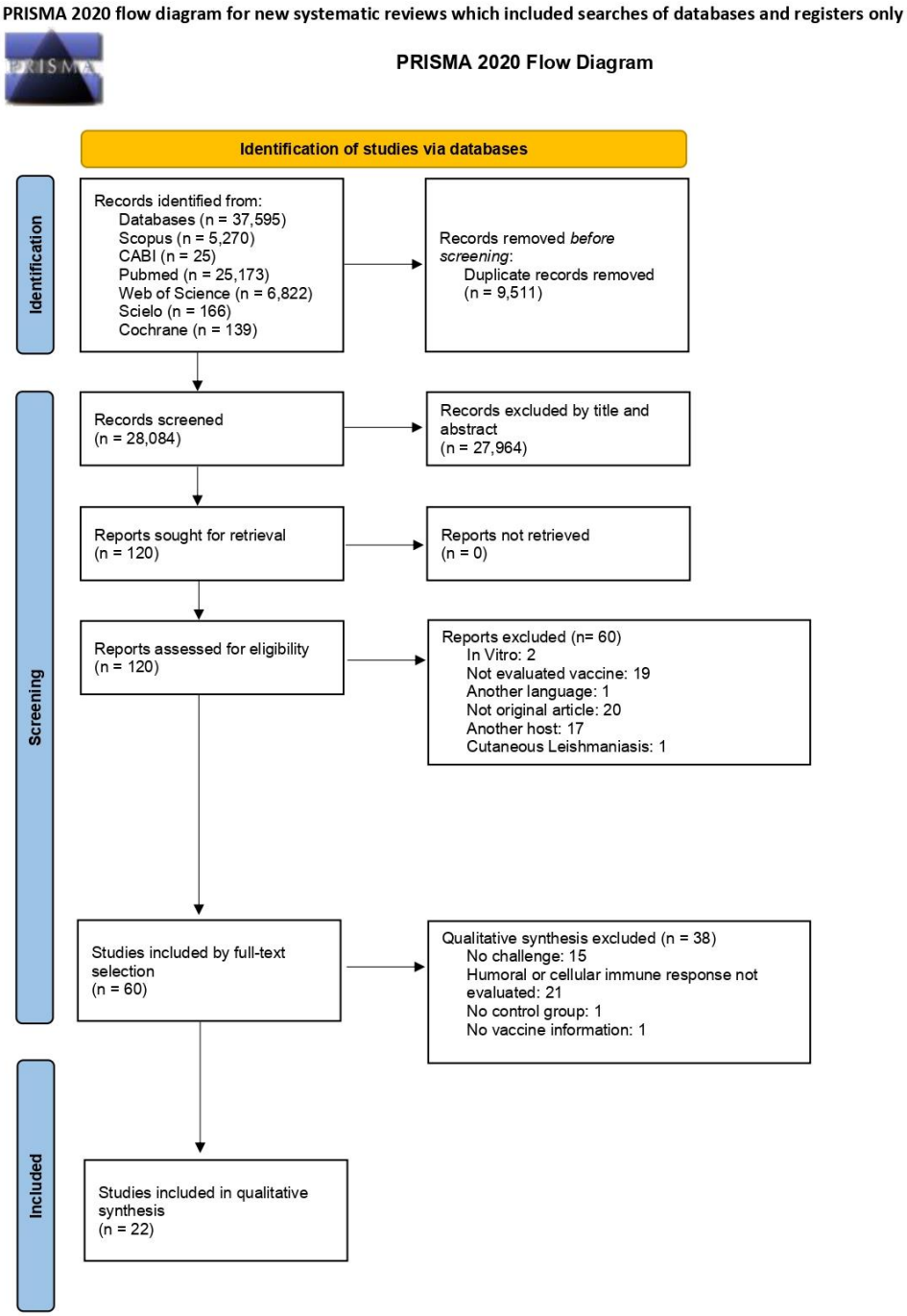


Table 1 – General characteristics of studies includes in the systematic review "Efficacy of vaccines against CVL"

First author, year	Country	Type of study	Total number of animals	Additional vaccines[f]	Dog breeds	Sex	Age
Abbehusen, 2018[a]	Brazil	Intervention[d]	30	Group 1[h]	Beagle	Both[l]	2-3 m[m]
Aguiar-Soares, 2014	Brazil	Intervention[d]	20	Group 1[h]	UB[k]	Both[l]	7- 8 m[m]
Alcolea, 2019	Spain	Intervention[d]	10	UN[g]	Beagle	Both[l]	12-18 m[m]
Borja-Cabrera, 2009[b]	Brazil	Intervention[d]	19	Group 1[h]	UB[k]	Both[l]	4 m[m]
Bourdoiseau, 2009[b]	France	Intervention[d]	12	UN[g]	UN[g]	Both[l]	UN[g]
Carcelen, 2009	Spain	Intervention[d]	21	UN[g]	Beagle	Both[l]	12-24 m[m]

De Lima, 2010	Iran	NC[e]	40	UN[g]	UN[g]	Both[l]	UN[g]
Fernandes, 2008	Brazil	Intervention[d]	21	Group 1[h]	Beagle	Both[l]	3-9 m[m]
Fiuza, 2015	Brazil	Intervention[d]	18	UN[g]	Beagle	Both[l]	8 m[m]
Gradoni, 2005	Brazil	NC[e]	45	Group 1[h]	Beagle	Both[l]	6 m[m]
Lemesre, 2005	France	Intervention[d]	18	UN[g]	Beagle	Both[l]	12-72 m[m]
Lemesre, 2007	France	NC[e]	414	UN[g]	UN[g]	Both[l]	UN[g]
Martin, 2014	France	Intervention[d]	20	UN[g]	Beagle	Both[l]	6 m[m]

Petitdidier, 2016	France	Intervention[d]	19	Routine vaccines	Beagle	Both[l]	24-48 m[m]
Petitdidier, 2019	France	Intervention[d]	15	Routine vaccines	Beagle	Both[l]	24-48 m[m]
Poot, 2009	Germany	Intervention[d]	44	UN[g]	Beagle	Both[l]	6 m[m]
Poot, 2006	France	Intervention[d]	15	UN[g]	Beagle	Males	6 m[m]
Ramiro, 2003	Spain	Intervention[d]	20	Group 2[i]	Beagle	Both[l]	18-54 m[m]
Roatt, 2012	Brazil	Intervention[d]	20	Group 1[h]	UB[k]	Both[l]	UN[g]
Rodriguez-Cortés, 2007	Spain	Intervention[d]	12	Group 3[j]	Beagle	Females	9 m[m]

Shahbazi, 2015	Iran	Intervention[d]	30	Group 1[h]	UB[k]	Both[l]	6-48 m[m]
Velez, 2020[c]	Spain	NC[e]	168	UN[g]	UN[g]	Both[l]	>6[n] m[m]

[a]This study has been corrected and its errata were also considered in this review. [b]Only some data were taken from this study, which fit the quality criteria. [c]In this article, the number of animals considered were the ones that ended up as vaccinations and did all the tests. [d] Studies that performed experimental infection in dogs. [e]NC: Natural challenge. [f]Other vaccines given to animals other than CVL. [g]UN: Uninformed; [h]Group 1: Vaccines group composed of rabies, distemper, hepatitis, adenovirus, leptospirosis, parvovirus, coronavirus; [i]Group 2: Distemper, Leptospirosis, Adenovirus, Hepatitis, Parainfluenza and Parvovirus; [j]Group 3: Distemper, Leptospirosis, Hepatitis, Parainfluenza and Parvovirus; [k]UB: Undefined breed. [l]Both sexes. [m]m: Age defined in months. [n]>6– More than 6 months.

Figure 2- Risk of bias summary: Authors' judgments of each risk of bias item for each included study, based on SYRCLE's RoB tool.

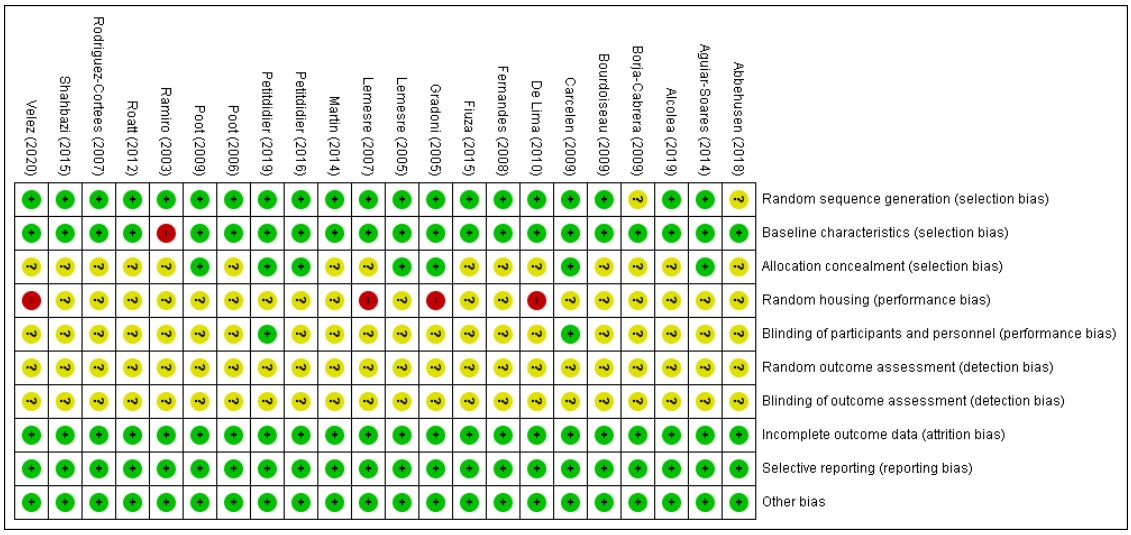
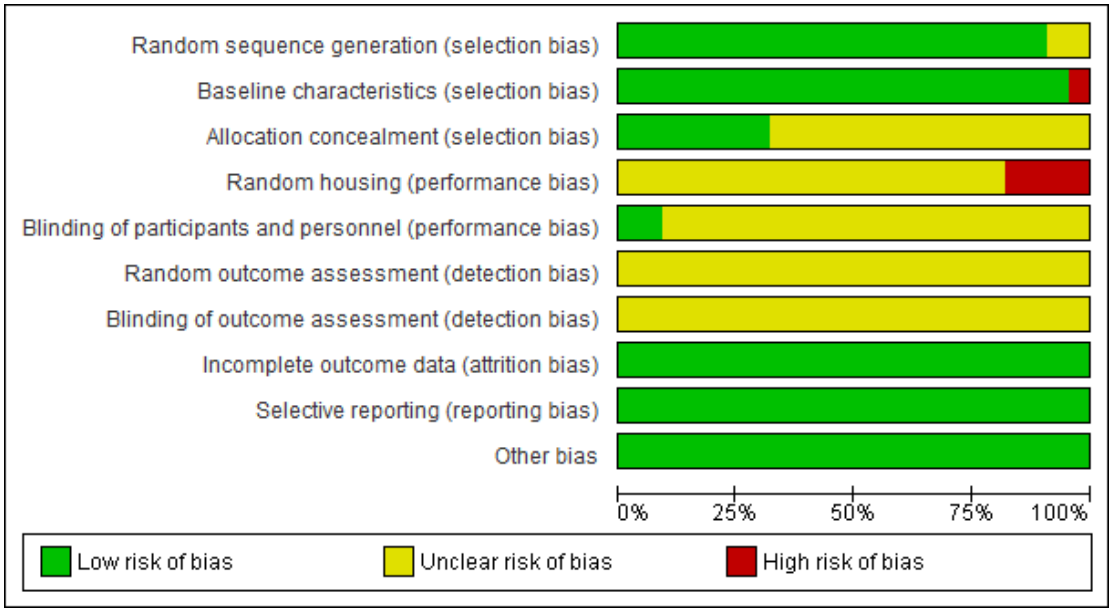


Figure 3 - Risk of bias graph: Author's judgments about each risk of bias item based on in SYRCLE's RoB tool, presented as percentages across all included studies.



3.2- Vaccine, dose and route

Of the selected studies, 81.8% (18/22) underwent experimental infection and only 18.2% (4/22) performed a natural challenge, in which the evaluated animals were exposed to the parasite, in the environment in which they lived. Individuals of both sexes were used in 90.9% (20/22) of the studies, and only one article (Rodriguez-Cortés et al., 2007) used only females in their groups, with the number of dogs per group varying between 3 and 85 and ages ranging from 2 to 54 months. The vaccinated and control groups were predominantly composed of beagle animals, used in 63.64% (14/22) of the studies.

Most studies, 84.4% (38/45), tested second-generation vaccines and 15.6% (7/45) tested third-generation vaccines. However, among those selected in this review, there were no articles that tested first-generation vaccines, consisting of the inactivated or attenuated protozoan. The most tested antigens among the selected trials were the so-called *L. infantum* secreted/excreted antigens (LiESAp), together with MDP adjuvants (Bourdoiseau et al., 2009; Lemesre et al., 2005), QA-21 (Martin et al., 2014) or saponin (Velez et al., 2020). The effectiveness of Q protein (Carcelén et al., 2009), recombinant A2 antigen (Fernandes, et al., 2008), and FML (De Lima et al., 2010; Fiuza et al., 2015) was also tested. *Leishmania* antigen-activated C - kinase antigen (LACK) has been tested in different ways by different studies (Alcolea et al., 2019; Ramiro et al., 2003; Rodriguez-Cortés et al., 2007), as well as many others, presented in Table 2. Only 4.44% (2/45) trials applied only one dose of its immunogen (Carcélen et al., 2009; Fiuza et al., 2015) and only 2.22% (1/45) (Rodriguez-Cortés et al., 2007) performed four applications. Most trials performed two or three applications, with intervals varying between 15 and 28 days (Supplementary Table S5) and the most common route of administration was subcutaneous, used in 88.9% (40/45) of the time (Table 2).

3.3- Methods to confirm immunogenicity

To confirm the immunogenicity of the antigens, the studies carried out several tests. To assess cellular immune response, 5.55% (1/18) of the studies that performed experimental infection (Figure 4) used the Enzyme-linked immunosorbent assay (ELISA) and 61.11% (11/18) used RT- qPCR, in order to evaluate cytokines such as IL-10, INF-γ and IL-4 in materials such as animal serum and cell culture supernatant. For the evaluation of cell types present in culture, Flow Cytometry was used in 38.89% (7/18) of the studies. 27.78% (5/18) studies also evaluated the production of nitric oxide (NO) and the Canine Macrophage Leishmanicidal Assay (CMLA). The studies that performed natural challenges also used the same tests to evaluate the cellular immune response to the antigen (Figure 5). For analysis of

humoral immune response, antibody titers in animal sera were evaluated through ELISA tests, used in 100% of experimental infection studies (Supplementary Table S6) and natural infection (Supplementary Table S7) and IFAT, performed in 50% (2/4) studies (Carcélen et al., 2009; Martin et al., 2014).

Figure 4 - Percentage of tests used by studies that performed experimental infection to evaluate cellular immune response against–vaccinal antigen.

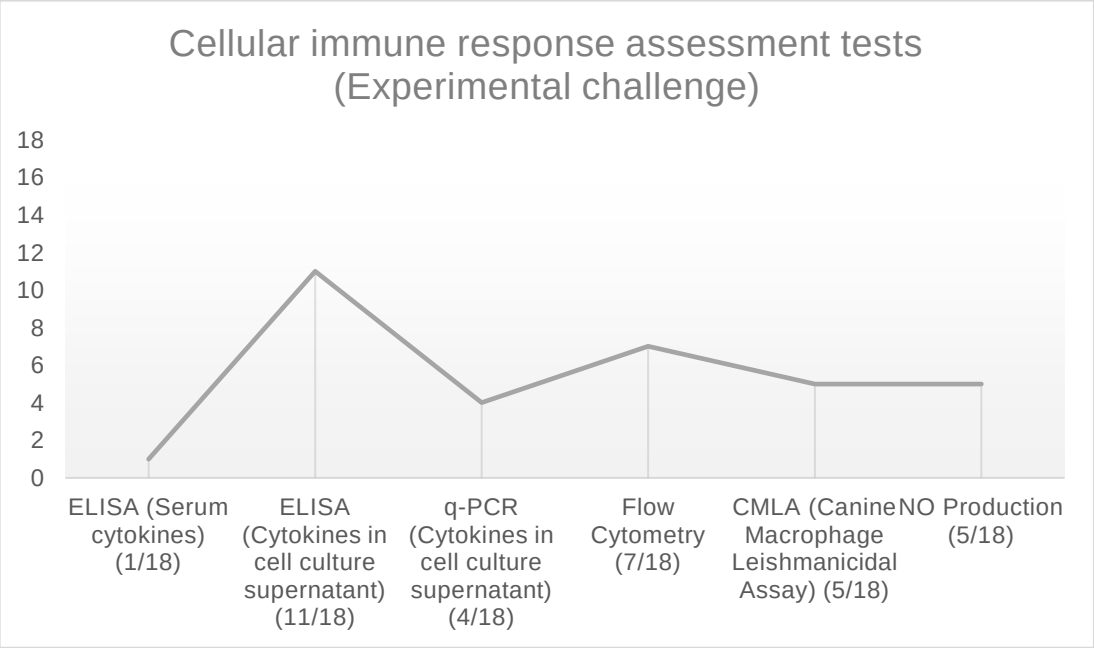
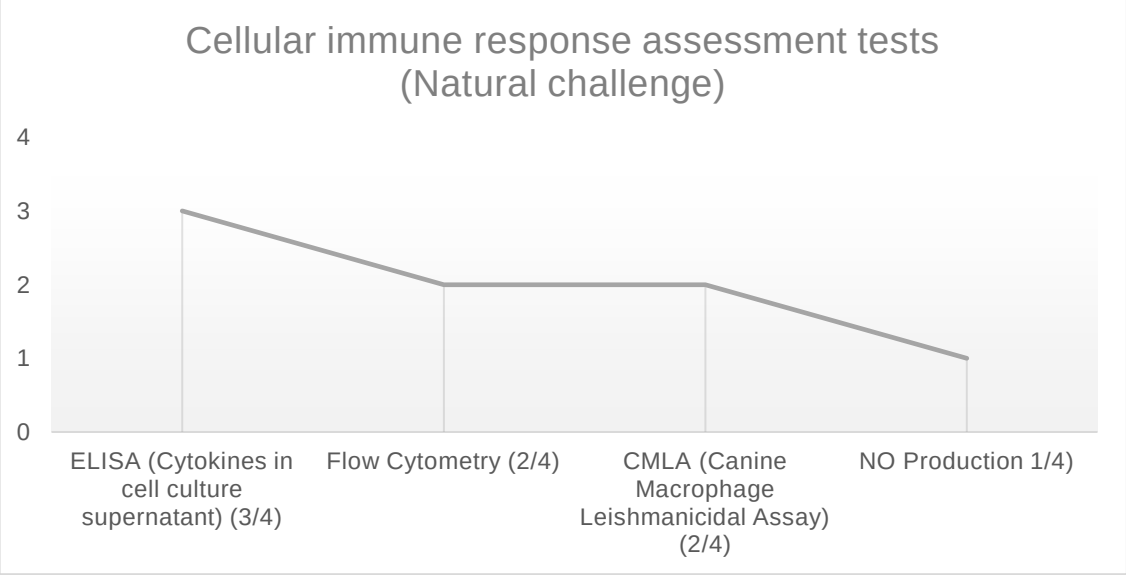


Figure 5 - Percentage of tests used by studies that performed natural infection to evaluate cellular immune response against vaccinal antigen.



3.4- Challenge strains, dose and route of exposure

To perform the efficacy rate calculations of the tested vaccines, the studies performed experimental or natural challenges of their vaccinated and control groups. All experimental infection studies used promastigotes of *L. infantum* in the challenge (Table 2), in more common doses of $5x10^7$ parasites, in 28.9% (13/45) of the trials, 10^8 parasites, in 26.7% (12/45) of the trials, and 10^7 parasites, in 17.8% (8/45) of trials. The most used route to perform the challenge was intravenous and the periods ranged from 0 to 240 days after the last vaccination. Among those who underwent natural infection, the exposure periods varied between 30 and 720 days after the last dose of immunogen was administered to the animals.

Table 2 – Vaccination and challenge data from trials selected for systematic review "Efficacy of vaccines against CVL"

First author, year	Vaccine's generation	N Vac[c]	N C[d]	Control Group	Vaccination				Challenge		
					Antigen/Adjuvant	Age (Months)	Route	Type of Challenge	Strain	Dose[s]	Route
Abbehusen, 2018[u]	3a[a]	10	10	P[x]	LJM17	2-3	IM[m]	E[o]	LIP[q]	10^7	ID[l]
Abbehusen, 2018[u]	3a[a]	10	10	P[x]	LJL143	2-3	IM[m]	E[o]	LIP[q]	10^7	ID[l]
Aguiar-Soares, 2014	2a[b]	5	5	P[x]	LB[f] + SGE[g]/Saponin	7- 8	SC[k]	E[o]	LIP[q]	10^7	ID[l]
Aguiar-Soares, 2014	2a[b]	5	5	P[x]	LB[f] + SGE[g]	7-8	SC[k]	E[o]	LIP[q]	10^7	ID[l]
Aguiar-Soares, 2014	2a[b]	5	5	P[x]	SGE[g]	7-8	SC[k]	E[o]	LIP[q]	10^7	ID[l]
Alcolea, 2019	3a[a]	5	5	P[x]	pPAL-LACK	12-18	IN[n]	E[o]	LIP[q]	10^8	UN[e]
Borja-Cabrera, 2009	3a[a]	6	13	P[x]	VR1012-NH36	4	IM[m]	E[o]	LIA[r]	$7x10^8$	UM
Bourdoiseau, 2009[v]	2a[b]	3	3	A[y]	LiESAp/MDP	UN[e]	SC[k]	E[o]	LIP[q]	10^8	ID[l]
Bourdoiseau, 2009[v]	2a[b]	3	3	A[y]	LiESAp/MDP	UN[e]	SC[k]	E[o]	LIP[q]	10^8	ID[l]
Carcelen, 2009	2a[b]	7	7	P[x]	Q-Protein	12-24	SC[k]	E[o]	LIP[q]	10^5	IV[t]
Carcelen, 2009	2a[b]	7	7	P[x]	Q-Protein	12-24	SC[k]	E[o]	LIP[q]	10^5	IV[t]
De Lima, 2010	2a[b]	20	20	P[x]	FML	UN[e]	SC[k]	NC[p]	NC[p]	NC[p]	NC[p]
De Lima, 2010	2a[b]	20	20	P[x]	FML	UN[e]	SC[k]	NC[p]	NC[p]	NC[p]	NC[p]
Fernandes, 2008	2a[b]	14	7	P[x]	rA2/Saponin	3-9	SC[k]	E[o]	LIP[q]	$5x10^7$	IV[t]
Fernandes, 2008	2a[b]	14	7	P[x]	rA2/Saponin	3-9	SC[k]	E[o]	LIP[q]	$5x10^7$	IV[t]
Fernandes, 2008	2a[b]	14	4	P[x]	rA2/Saponin	3-9	SC[k]	E[o]	LIP[q]	$5x10^7$	IV[t]
Fernandes, 2008	2a[b]	14	3	A[y]	rA2/Saponin	3-9	SC[k]	E[o]	LIP[q]	$5x10^7$	IV[t]
Fiuza, 2015	2a[b]	6	6	P[x]	LdCen	8	SC[k]	E[o]	LIP[q]	10^7	IV[t]
Fiuza, 2015	2a[b]	6	6	P[x]	FML	8	SC[k]	E[o]	LIP[q]	10^7	IV[t]
Gradoni, 2005	2a[b]	15	15	P[x]	MML/ MPL-SE	6	SC[k]	NC[p]	NC[p]	NC[p]	NC[p]
Gradoni, 2005	2a[b]	15	15	P[x]	MML/ Adjuprime	6	SC[k]	NC[p]	NC[p]	NC[p]	NC[p]
Lemesre, 2005	2a[b]	3	3	A[y]	LiESAp/MDP	12-72	SC[k]	E[o]	LIP[q]	10^8	IV[t]
Lemesre, 2005	2a[b]	3	3	A[y]	LiESAp/MDP	12-72	SC[k]	E[o]	LIP[q]	10^8	IV[t]

Lemesre, 2005	2a[b]	3	3	A[y]	LiESAp/MDP	12-72	SC[k]	E[o]	LIP[q]	10^{8}	IV[t]
Lemesre, 2005	2a[b]	3	3	A[y]	LiESAp/MDP	12-72	SC[k]	E[o]	LIP[q]	10^{8}	IV[t]
Lemesre, 2007	2a[b]	UN[e]	UN[e]	P[x]	LiESAp/MDP	UN[e]	SC[k]	NC[p]	NC[p]	NC[p]	NC[p]
Martin, 2014	2a[b]	10	10	P[x]	LiESP/QA-21	6	SC[k]	E[o]	LIP[q]	$10^{8,5}$	IV[t]
Petitdidier, 2016	2a[b]	9	5	P[x]	LaPSA-38S/QA-21	24-48	SC[k]	E[o]	LIP[q]	10^{8}	IV[t]
Petitdidier, 2016	2a[b]	5	5	P[x]	LaPSA-12S/QA-21	24-48	SC[k]	E[o]	LIP[q]	10^{8}	IV[t]
Petitdidier, 2019	2a[b]	10	5	P[x]	A17G + A17E + E34PC/QA-21	24-48	SC[k]	E[o]	LIP[q]	10^{8}	IV[t]
Poot, 2009	2a[b]	7	7	P[x]	rJPCM5_Q[h]/ MDP	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Poot, 2009	2a[b]	7	7	P[x]	rJPCM5_Q[h]/ Aluminum hydroxide	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Poot, 2009	2a[b]	7	7	P[x]	rJPCM5_Q[h]/ ISCOMatrix C	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Poot, 2009	2a[b]	5	1	P[x]	rJPCM5_Q[i]/ MDP	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Poot, 2009	2a[b]	5	1	P[x]	rJPCM5_Q[i]/ Aluminum hydroxide	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Poot, 2009	2a[b]	5	1	P[x]	rJPCM5_Q[i]/ ISCOMatrix C	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Poot, 2006	2a[b]	5	5	P[x]	rCPA + rCPB/ rIL-12	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Poot, 2006	2a[b]	5	5	P[x]	rCPA + rCPB/ rIL-12 + QuilA	6	SC[k]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Ramiro, 2003	3a[a]	5	5	P[x]	DNA-LACK	18-54	SC[k]	E[o]	LIP[q]	10^{8}	IV[t]
Ramiro, 2003	3a[a]	5	5	P[x]	DNA-LACK + rVV-LACK	18-54	SC[k]	E[o]	LIP[q]	10^{8}	IV[t]
Roatt, 2012	2a[b]	5	5	P[x]	LBf/Saponin	UN[e]	SC[k]	E[o]	LIP[q]	10^{7}	ID[l]
Rodriguez-Cortés, 2007	3a[a]	6	6	P[x]	pMOK-Kmp11/-TRYP/-LACK/-GP63	9	ID[l]	E[o]	LIP[q]	$5x10^{7}$	IV[t]
Shahbazi, 2015	3a[a]	10	10	P[x]	pcDNA-A2-CPACPB−CTEGF P (cSLN)	6-48	SC[k]	E[o]	LIP[q]	$4x10^{7}$	IV[t]
Shahbazi, 2015	3a[a]	10	10	P[x]	pcDNA-A2-CPACPB−CTEGFP (Electroporation)	6-48	SC[k]	E[o]	LIP[q]	$4x10^{7}$	IV[t]

Velez, 2020[w]	2a[b]	85	83	P[x]	LiESP/Saponin	>6[j]	SC[k]	NC[p]	NC[p]	NC[p]	NC[p]

[a]3a: Third Generation; [b]2a: Second Generation; [c]N Vac: number of vaccinated animals; [d]N C: number of control animals; [e]UN: Uninformed; [f]LB: *L. braziliensis* protein; [g]SGE: Sand fly salivary gland extract; [h]rJPCM5_Q: Antigen produced by *E. coli;* [i]rJPCM5_Q: Antigen produced by *Baculovirus;* [j]>6: More than 6 months; [k]SC: subcutaneous; [l]ID: intradermal, [m]IM: intramuscular; [n]IN: Intranasal; [o]E: Experimental challenge; [p]NC: Natural challenge; [q]LIP: *L. infantum* promastigotes; [r]LIA: *L. infantum* amastigotes; [s]Dose: Unity = Parasites; [t]IV: Intravenous. [u]This trial has been corrected and its errata were also considered in this review. [v]Only some data were taken from this trial, which fit the quality criteria. [w]In this article, the number of animals considered were the ones that ended up as vaccinations and did all the tests; [x]P: Placebo; [y]A: Adjuvant

3.5- Diagnostic methods to confirm infection and evaluation of clinical signs

To confirm the presence or absence of infection, the following tests were performed in the studies: q-PCR testing in 77.28% (17/22), ELISA in 22.7% (5/22), direct tissue visualization testing in 18.2% (4 /22), parasite culture, in 50% (11/22), IFAT in 9.1% (2/22) and DAT in 4.5% (1/22). Clinical evaluation was performed to detect infected, symptomatic, or asymptomatic animals, this evaluation was performed in 63.6% (14/22) of the studies. The main signs observed were skin and adnexal lesions (alopecia, ulcers, exfoliative dermatitis, onychogryphosis), nutritional status, eye lesions (uveitis, conjunctivitis, keratoconjunctivitis), and lymphadenopathy. These changes are commonly found in dogs with canine visceral leishmaniasis.

3.6- Assessment of protection against disease

The Canine Visceral Leishmaniasis Vaccine Efficacy Rate was calculated from data on symptomatic and asymptomatic dogs between the vaccinated and control groups. Most of the immunogens in these studies that carried out experimental infection did not obtain good rates of protection against the clinical signs of the disease (Table 3). However, the vaccine composed of LiESP /QA-21 (Martin et al., 2014) showed 62% (RR = 0.375, 95% CI: 0.13 – 0.88) of protection against LVC and the one composed of DNA-LACK, in the first dose, and revaccination with rVV -LACK (Ramiro et al., 2003) showed 80% (RR = 0.2, 95% CI: 0.03 – 0.66) protection against CVL. Among the experiments that performed the challenge and made it possible to perform the analyzes (Table 4), only one showed a significant protection rate. The immunogen composed of LiESAp added to the adjuvant MDP (Lemesre et al., 2007), showed 100% (RR = 0.95% CI: 0.00 – 0.88) protection against the disease, considering that none of the vaccinated animals showed clinical signs of the disease.

Table 3 – Efficacy of the vaccine antigen against the development of CVL in vaccinated and unvaccinated animals in studies that performed experimental challenge.

First author, year	Antigen/Adjuvant	Challenge dose	Asymptomatic animals[c]		Symptomatic animals[d]		Relative Risk	[e]CI (95%)	Vaccine Efficacy (1 - RR[f])
			NVac[a](%)	NC[b](%)	NVac[a](%)	NC[b](%)			
Abbehusen, 2018	LJM17	10^7	0	0	10/10 (100)	10/10 (100)	1	0.72-1.38	0
Abbehusen, 2018	LJL143	10^7	0	0	10/10 (100)	10/10 (100)	1	0.72-1.38	0
Alcolea, 2019	pPAL-LACK	10^8	1/5 (20)	1/5 (20)	4/5 (80)	4/5 (80)	1	0.44-2.25	0
Carcelen, 2009	Q-Protein	10^5	4/7 (57.15)	1/7 (14.3)	3/7 (42.9)	6/7 (85.7)	0.5	0.17-1.13	0.5
Carcelen, 2009	Q-Protein	10^5	2/7 (28.57)	1/7 (14.3)	5/7 (71.42)	6/7 (85.7)	0.833	0.40-1.59	0.16
Fernandes, 2008	rA2/Saponin	5x10^7	5/7 (71.42)	2/7 (28.57)	2/7 (28.,57)	5/7 (71.42)	0.4	0.10-1.20	0.6
Martin, 2014	LiESP/QA-21	10^8,5	7/10 (70)	2/10 (20)	3/10 (30)	8/10 (80)	0.375	0.13-0.88	0.62

Ramiro, 2003	DNA-LACK	10^8	0	0	5/5 (100)	5/5 (100)	1	0.56-1.76	0
Ramiro, 2003	DNA-LACK + rVV-LACK	10^8	4/5 (80)	0	1/5 (20)	5/5 (100)	0.2	0.03-0.66	0.8
Rodriguez-Cortés, 2007	pMOK-Kmp11/-TRYP/-LACK/-GP63	5x10^7	4/6 (66.67)	2/6 (33.33)	2/6 (33.33)	4/6 (66.67)	0.5	0.13-1.58	0.5

[a]N Vac: Number of vaccinated animals; [b]N C: Number of control animals; [c]Animals without clinical signs; [d]Animals with clinical signs; [e]Confidence interval; [f]RR: Relative Risk.

Table 4 – Efficacy of the vaccine antigen against the development of CVL in vaccinated and unvaccinated animals in studies that performed natural challenge.

First author, year	Antigen/Adjuvant	Time of exposition (years)	Asymptomatic animals[c]		Symptomatic animals[d]		Relative Risk	[e]CI (95%)	Vaccine Efficacy (1 - RR[f])
			NVac[a](%)	NC[b](%)	NVac[a](%)	NC[b](%)			
Gradoni, 2005	MML + MPL-SE	1	6/6 (100)	6/6(100)	0	0	I[g]	I[g]	I[g]
Gradoni, 2005	MML + Adjuprime	1	5/5 (100)	6/6(100)	0	0	I[g]	I[g]	I[g]
Gradoni, 2005	MML + MPL-SE	2	10/13 (76.92)	14/14(100)	3/13 (23.07)	0	I[g]	I[g]	I[g]
Gradoni, 2005	MML + Adjuprime	2	8/10 (80)	14/14(100)	2/10 (20)	0	I[g]	I[g]	I[g]
Lemesre, 2007	LiESAp + MDP	2	8/8 (100)	7/17(41.17)	0	5/17 (29.41)	0	0.00-0.88	1

[a]N Vac: Number of vaccinated animals; [b]N C: Number of control animals; [c]Animals without clinical signs; [d]Animals with clinical signs; [e]Confidence interval; [f]RR: Relative Risk; [g]Impossible to calculate

3.7- Assessment of protection against infection

The vaccine efficacy rate against *L. infantum* infection was calculated from data from dogs with a confirmed infection by at least one diagnostic method performed by the study, between the vaccinated and control groups. Among the studies that performed experimental infection (Table 5), only one showed a significant result. The experiment carried out with the immunogen DNA-LACK, administered to the animals in the first dose and with the immunogen rVV-LACK, administered in the subsequent doses, showed 60 % (RR = 0.4, 95% CI: 0.11 – 0.93) efficacy against infection by *L. infantum* (Ramiro et al., 2003). On the other hand, studies that performed a natural challenge (Table 6) did not demonstrate significant protection against infection.

Table 5 – Efficacy of the vaccine antigen against *L. infantum* infection in vaccinated and unvaccinated animals in studies that performed experimental challenge.

First author, year	Antigen/Adjuvant	Challenge dose	Positive Animals[c]		Negative Animals[d]		Relative Risk	[e]CI (95%)	Vaccine Efficacy (1 - RR[f])
			NVac[a](%)	NC[b](%)	NVac[a](%)	NC[b](%)			
Abbehusen, 2018	LJM17	10^7	10/10 (100)	10/10 (100)	0	0	1	0.72-1.38	0
Abbehusen, 2018	LJL143	10^7	10/10 (100)	10/10 (100)	0	0	1	0.72-1.38	0
Alcolea, 2019	pPAL-LACK	10^8	5/5 (100)	5/5(100)	0	0	1	0.56-1.76	0
Carcelen, 2009	Q-Protein	10^5	5/7 (71.4)	7/7 (100)	2/7 (28.57)	0	0.7143	0.35-1.18	0.2857
Carcelen, 2009	Q-Protein	10^5	6/7 (85.71)	7/7 (100)	1/7 (14.28)	0	0.8571	0.48-1.36	0.1429
Fernandes, 2008	rA2/Saponin	5x10^7	4/7 (57.15)	7/7 (100)	3/7 (42.85)	0	0.5714	0.25-1.0	0.4286
Martin, 2014	LiESP/QA-21	10^8,5	7/10 (70)	8/10 (80)	3/10 (30)	2/10 (20)	0.875	0.47-1.54	0.125
Ramiro, 2003	DNA-LACK	10^8	5/5 (100)	5/5 (100)	0	0	1	0.56-1.76	0
Ramiro, 2003	DNA-LACK + rVV-LACK	10^8	2/5 (40)	5/5 (100)	3/5 (60)	0	0.4	0.11-0.93	0.6
Rodriguez-Cortees, 2007	pMOK-Kmp11/-TRYP/-LACK/-GP63	5x10^7	6/6 (100)	2/6 (33.33)	0	4/6 (66.67)	I[g]	I[g]	I[g]

[a]N Vac: Number of vaccinated animals; [b]N C: Number of control animals; [c]Positive animals in at least one diagnostic test performed by the study; [d]Negative animals in all diagnostic tests performed by the study; [e]Confidence interval; [f]RR: Relative Risk; [g]Impossible to calculate

Table 6 – Efficacy of the vaccine antigen against *L. infantum* infection in vaccinated and unvaccinated animals in studies that performed natural challenge.

First author, year	Antigen/Adjuvant	Time of exposition (years)	Positive Animals[c]		Negative Animals[d]		Relative Risk	[e]CI (95%)	Vaccine Efficacy (1 - RR[f])
			NVac[a](%)	NC[b](%)	NVac[a](%)	NC[b](%)			
Gradoni, 2005	MML + MPL-SE	1	6/15 (40)	6/14 (43)	9/15 (60)	8/14 (57)	0.9333	0.39-2.22	0.0667
Gradoni, 2005	MML + Adjuprime	1	5/14 (56)	6/14 (43)	9/14 (64)	8/14 (57)	0.8333	0.32-2.06	0.1667
Gradoni, 2005	MML + MPL-SE	2	13/15 (87)	14/14 (100)	2/15 (13.33)	0	0.8667	0.62-1.12	0.1333
Gradoni, 2005	MML + Adjuprime	2	10/10 (100)	14/14 (100)	0	0	1	0.72-1.27	0
Lemesre, 2007	LiESAp + MDP	2	7/11 (63,64)	14/17 (82,35)	4/11 (36,36)	3/17 (17.64)	0.7727	0.41-1.21	0.2273

[a]N Vac: Number of vaccinated animals; [b]N C: Number of control animals; [c]Positive animals in at least one diagnostic test performed by the study; [d]Negative animals in all diagnostic tests performed by the study; [e]Confidence interval; [f]RR: Relative Risk;

4- Discussion

This systematic review aimed to recalculate vaccine efficacy rates against Canine Visceral Leishmaniasis, as well as against *L. infantum* infection in dogs. The selected studies carried out their experiments between 2003 and 2020, in several countries, with a predominance of Brazil and France, both with 31.82% (7/22) of the studies each. It is important to develop studies such as these, which look for advances in relation to vaccines, since it is a complicated task to obtain an immunogen capable of inducing a strong and lasting specific protective response against pathogens of high antigenic complexity, such as protozoa of genus *Leishmania* (Moreno, 2019).

In order to select studies that contained complete information about the trials, quality criteria were defined so that they would be eligible for the analysis of this review. One of the most important criteria adopted was the presence of control groups so that there could be assertive comparisons with the vaccinated animals. Most of the selected experiments used substances such as a saline solution in their control groups, and some, in addition to placebo, used the adjuvant. The use of the adjuvant as a control group is quite valid, as it is of great value to know how adjuvants contribute to the protection of a vaccine, since their physicochemical properties, as well as their doses, routes, and frequency of administration, can directly influence the quality of the Immune response, mainly stimulating the innate immune response, which will define the characteristics and extent of the adaptive response (Batista-Duharte et al., 2018). However, studies that use only adjuvants as a control, as in two of the selected studies (Bourdoiseau et al., 2009; Lemesre et al., 2005), do not use the best strategy, since a comparison of responses with animals that did not receive substances capable of stimulating the immune system, so that they can predict what are the results attributable to the presence of the antigen added to the adjuvant and to the adjuvant alone.

In order to obtain sufficiently reliable information to be used in this review, the studies were also submitted to the risk of bias analysis, using SYRCLE''s Rob Tool. This tool seeks to guide authors of systematic reviews involving studies with animals and has very valid criteria, which studies that seek to calculate vaccine efficacy should pay attention to (Hooijmans et al., 2014). However, it is necessary to point out that this tool is aimed at the evaluation of studies in mice and, therefore, may not be the most suitable for evaluating studies involving dogs. The issue of random spatial allocation of the animals involved in the experiment, for example, is something quite relevant to be considered and is highly emphasized by the tool, as vertebrates have the ability to regulate various physiological processes through the circadian cycle, which directly interferes with its neuronal activities, causing the production of hormones that can

affect the entire body, including the functioning of the immune system (Downton et al., 2019). However, it is possible to observe that 81.8% (18/22) of the studies did not contain information on random housing of dogs, demonstrating that often these conditions, which are quite easy to apply to mice, cannot be applied in the same way to dogs, for costs reasons, logistics and other factors. Therefore, it is necessary to emphasize that, even with uncertain or high bias for some domains of the SYRCLE tool, the works evaluated here were considered of good quality, since much of what is evaluated in the tool is very well applicable to studies with mice, but for dogs may be unfeasible to perform. This demonstrates the need to create a specific tool to assess the risk of bias aimed at studies with dogs, with domains more suited to the reality of research with these animals.

The excreted/secreted antigens of *L. infantum*, despite having been tested in several studies, showed protection against CVL in only one of the studies (Martin et al., 2014). The vaccine composed of LiESP + QA-21, administered in three vaccinations, with 100 μg of the antigen at 21-day intervals between applications, showed 62% (RR = 0.375, 95% CI: 0.13 – 0.88) of protection against CVL, but did not provide considerable protection against infection, which is common among vaccines against *Leishmania* spp. Another study of experimental infection (Bourdoiseau et al., 2009) tested this antigen in a similar protocol, with the same dosages, frequencies, and intervals of administration, however, it did not evaluate clinical signs and therefore, it was not possible to compare the protection rates between them. Failure to assess clinical signs also made it impossible to compare the study by Martin et al. (2014) and the essays performed by Lemesre et al. (2005) who tested *L. infantum* excreted/secreted antigens in two applications with 21-day intervals, at doses of 50 μg, 100 μg, and 200 μg. The absence of an evaluation of clinical signs made it impossible to analyze the rate of vaccine efficacy against CVL in several studies included in this review (Aguiar-Soares et al., 2014; Fiuza et al., 2015; Petitdidier et al., 2016; Roatt et al., 2012). The observation of clinical parameters for studies involving vaccine efficacy is relevant, since the clinical evaluation combined with laboratory tests allows a more accurate diagnosis, in addition to indicating an animal with a greater possibility of transmitting the protozoan to the sand fly, considering that dogs Symptomatic individuals have, on average, a higher parasite load in the skin and other tissues, when compared to asymptomatic individuals (Torcilha et al., 2016).

The LACK vaccine antigen, tested by Ramiro et al., 2003, is administered in two applications, the first with the antigen in a plasmid vector at a dose of 100 μg and the second with a recombinant viral vector (rVV -LACK) at a dose of 10^8 PFU, with an interval of 15 days

between applications, showed 80% (RR = 0.2, 95% CI: 0.03 – 0.66) of protection against LVC. This vaccine was the only one, among those tested by experimental infection trials, to show protection [60% (RR = 0.4, 95% CI: 0.11 – 0.93)] against *L. infantum* infection. This data is relevant because, to become an efficient collective prevention measure, with a relevant impact on the number of human cases of Leishmaniasis, it is necessary that, in addition to preventing the onset of clinical signs of CVL, the immunogen can prevent infection, so that the animals stop transmitting the parasite to the sandfly. However, a partial efficacy rate is not enough to fulfill this role, as already demonstrated by studies that evaluate commercially available vaccines in Brazil and Europe (Dantas-Torres et al., 2020). The same study carried out a test in another group of dogs, with the same antigen, but with only one dose of LACK in a plasmid vector. All animals (5/5) in this group, at the end of the evaluations, were infected and symptomatic, as was the case in the control group, indicating that this antigen, in this vehicle, in a single application, was not sufficiently effective in combating the infection or disease, requiring two doses of the antigen to result in beneficial effects. Other studies (which tested the same antigen, however, in different vehicles, doses, and frequencies of administration, did not show significant results (Alcolea et al., 2019; Rodriguez-Cortés et al., 2007).

Studies that performed natural exposure tested FML antigens (De Lima, et al., 2010), Leishmune® vaccine composition, MML (Gradoni et al., 2005), added to MDP adjuvant, LiESAp (Lemesre et al., 2007) and LiESP (Velez et al., 2020) added to saponin adjuvant. The author's study De Lima et al. (2010), was carried out in Araçatuba – Brazil. This location, in 2020, had a prevalence of 8% (95%CI: 6-10), according to Costa et al. (2020). However, it was not possible to assess the rates of protection against the disease, as the study did not assess clinical parameters, which is necessary for studies with visceral leishmaniasis vaccines. The study by Gradoni et al. (2005), carried out their experiments in northeastern Italy, more specifically in Montichiari, Brescia, where the prevalence of CVL in northeastern Italy in 2018 was around 2%, according to Rugnia et al. (2018), and evaluated their animals at intervals of up to 2 years after vaccination, in which 23.07% (3/13) of those vaccinated with MML + MPL-SE and 20% (2/10) of those vaccinated with MML + Adjuprime had clinical signs. Due to the absence of symptoms among the animals in the control group, statistical calculations were impossible and, therefore, it was not possible to calculate rates of vaccine protection against the disease in these trials. The study by Velez et al. (2020), in which the CaniLeish® vaccine was tested, despite having carried out the evaluation of clinical signs, presented data in a confusing way, in tables from which it was not possible to identify the data clearly, making extraction impossible and, therefore, the calculation of vaccine efficacy rates. The study by Lemesre et al.

(2007), who carried out their studies in the South of France, showed a 100% (RR = 0.95% CI: 0.00 – 0.88) rate of vaccine protection against the disease, however, there was no significant protection rate against the infection.

Other important information, framed in the quality criteria of this review, addressed the experimental challenges. It was necessary that the route, dose, and parasitic form used, as well as the post-vaccination time in which the challenge was carried out, were well described in the studies, considering the relevance of carrying out the exposure of dogs to the parasite for studies that aim to establish the effectiveness of a vaccine antigen. All experimental challenges were performed with promastigotes of *L. infantum*, as this species has a well-defined zoonotic cycle, with the important participation of dogs, especially in countries such as Brazil, where the prevalence of the disease is quite high in animals and humans (Marcondes & Day, 2019). The doses used ranged from 10^5 to $7x10^8$ parasites, administered intravenously in 74.4% (29/39) of the trials. These challenge protocols do not mimic the reality of natural infection and reveal the need for researchers to provoke the disease in a shorter time, since high amounts of parasites like these, intravenously, bring a greater guarantee of infection in animals. However, this could be harmful to the study, since the high number of parasites, inoculated by such an invasive route, can make the protection conferred by the vaccine to be hidden by the response to such an aggressive challenge. Ideally, studies such as these would mimic natural conditions as much as possible and to standardize the protocols used as much as possible, reducing the heterogeneity between them, to improve repeatability and the possibility of other researchers finding similar and more reliable results. For this, the intradermal route can be used, as occurred in 20.5% (8/35) of experimental infection trials (Roatt et al., 2012; Bourdoiseau et al., 2009; Aguiar-Soares et al., 2014; Abbehusen et al., 2018) and with a dose of parasites that is close to the dose inoculated by the sandfly in a real situation, ranging from 10,000 to 40,000 amastigotes. Therefore, it can be said that the studies that used the intravenous route may have underestimated the vaccine efficacy.

In conclusion, it can be seen that the antigens LiESP with QA-21 adjuvant, LACK, and LiESAp with the adjuvant MDP, were the only ones, among all the antigens of the studies analyzed in this review, to present significant protection rates. LACK antigen administered in two doses, even in different vectors in each application, managed to achieve protection against disease and infection. It is notable that this antigen, when tested in a single dose, was not able to reach significant levels of efficacy and, therefore, its administration in a double dose can be recommended, respecting a minimum interval of 15 days. The excreted/secreted antigens of *L. infantum* also showed considerable protection, both with the QA-21 adjuvant and with the MDP

adjuvant, provided that the doses of 100 μg of the antigen were respected, in three vaccinations, with a minimum interval of 21 days between applications, a protocol used by the two trials with significant protection rates. It is worth noting that the other experiments that evaluated *L. infantum* excreted/secreted antigens in different protocols did not achieve significant efficacy. Thus, the protocol adopted by Lemesre et al. (2007) and Martin et al. (2014), with an antigenic dose of 100 μg, in three vaccinations, with a minimum interval of 21 days, as the best applicable to this immunogen, and further studies are needed to corroborate this statement.

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Conflict of interests

The authors declare no competing interests.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a review article with no original research data.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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6- Supplementary tables

Supplementary Table S1 – Guidelines of PRISMA statement

Section/topic	#	Checklist item	Reported on §
		Title	
Title	1	Identify the report as a systematic review, meta-analysis, or both	§1
		Abstract	
Structured Summary	2	Provide s structured summary including, as applicable: background; objectives; data sources; study eligibility criteria; participants and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number	§1
		Introduction	
Rationale	3	Describe the rationale for the review in the context of what is already known	§1-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS)	§4
		Methods	
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	§1,2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	§2 Tab.[1] S2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	§1
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	§Tab. S2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	§3 Tab. S3
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	§5-6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	§4-6 Tab. S2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis	§6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	§7
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I2) for each meta-analysis.	§7
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	§6

Additional analysis	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	§7
Results			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	§1 Fig[2]. 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Tab. S4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	§1 Fig.2,3
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	§2-7 Tab. 1 – 6 Tab. S5-S6
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	-
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	-
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see item 16)]	-
Discussion			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers)	§1
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	§2 – 8
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	§9
Funding			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	§1

[1]Tab.: Table;[2]Fig.: Figure

Supplementary Table S2 – Search terms used in Pubmed, Web of Science, Scopus, Cochrane, Scielo and CABI, based on the PICOTS terms.

PICOTS	Search terms
Population	canine OR dog* OR pupp* OR (canis AND familiaris) OR animal*
Intervention	AND (Leishman*) OR CanL OR CVL OR Leish-Tec OR LetiFend OR rA2 OR "recombinant A2 antigen saponin" OR Leishmune OR FML OR "Fucose Mannose ligand saponin" OR LBSap OR Leish-111f OR LeishF1 OR "recombinant *Leishmania* polyprotein LEISH-F1 antigen" OR LiESAp OR LiESA-MDP OR CaniLeish OR LACK OR "*Leishmania* Homologue of Activated C Kinase" OR "Cysteine proteinases" OR H1 OR KMP-11 OR "Kinetoplastid membrane protein-11"
Comparison	prophyla* OR prevalen* OR persisten* OR incidenc* OR epidemiol* OR control* OR prevent* OR efficacy OR effect* OR immun* OR protect* OR safe* OR "therapeutic use"
Outcomes	antibod* OR serolog* OR "clinical signs" OR alopecia OR lymphadenomegaly OR hepatosplenomegaly OR onychogryphosis OR parasit* OR "parasite load" OR xenodiagnos*
Time	-
Setting	Systematic review

Supplementary Table S3 – Inclusion and exclusion criteria for selection of articles in this systematic review.

Inclusion criteria	Exclusion criteria
• All countries • All years • Canine visceral • Studies on vaccines • *L. infantum* • Vaccines tested on	• *In vitro* and *In silico* studies • Cohort studies • Case-control studies • Cross-sectional studies • Case reports and Reviews • Diagnostic performance of tests • Therapeutics • Languages other than English, Spanish or Portuguese • Full-text not available • No information about control group • No information about vaccine antigen, dose, route, • No challenge performed • No information about challenge dose, route, strain or • No information about evaluation of humoral immune • No information about evaluation of cellular immune • No information about parasite load • No information about vaccine protection rate

Supplementary Table S4 – Studies not selected by quality criteria in this review

First author, year	Reason
Araújo, 2011	No challenge
Araújo, 2009	No challenge
Bongiorno, 2013	Humoral or cellular immune response not evaluated
Borja-Cabrera,2012	Humoral or cellular immune response not evaluated
Borja-Cabrera, 2008	No control group
Borja-Cabrera, 2010	Humoral or cellular immune response not evaluated
Borja-Cabrera, 2002	Humoral or cellular immune response not evaluated
Cacheiro-Laguno, 2020	Humoral or cellular immune response not evaluated
Carillo, 2008	Humoral or cellular immune response not evaluated
Cotrina, 2018	Humoral or cellular immune response not evaluated
Daneshvar, 2010	No vaccine information
de Amorim, 2010	No challenge
Dunan, 1989	Humoral or cellular immune response not evaluated
Fallah, 1998	Humoral or cellular immune response not evaluated
Fernandes, 2014	Humoral or cellular immune response not evaluated
Fujiwara, 2005	Humoral or cellular immune response not evaluated
Giunchetti, 2007	No challenge
Giunchetti, 2008	No challenge
Giunchetti, 2008	No challenge
Grimaldi, 2017	Humoral or cellular immune response not evaluated
Vitoriano-Souza, 2008	Humoral or cellular immune response not evaluated
Lopes, 2018	Humoral or cellular immune response not evaluated
Martinez-Rodrigo, 2019	No challenge
Moreira, 2016	No challenge
Moreno, 2007	No challenge
Moreno, 2013	No challenge
Moreno, 2014	No challenge
Nogueira, 2005	Humoral or cellular immune response not evaluated
Oliva, 2014	Humoral or cellular immune response not evaluated
Silva, 2016	Humoral or cellular immune response not evaluated
Resende, 2016	Humoral or cellular immune response not evaluated
Resende, 2013	Humoral or cellular immune response not evaluated
Saraiva, 2006	Humoral or cellular immune response not evaluated
Testasicca, 2014	Humoral or cellular immune response not evaluated
Vannucci, 2018	No challenge
Velez, 2020	No challenge
Souza, 2013	No challenge
Ogunkolade, 1988	No challenge

Supplementary Table S5 – Detailed information about the vaccinations in the trials that performed this analysis among those selected by this systematic review.

			First vaccination		Second vaccination			Third vaccination			Fourth vaccination		
First author, year	**Route**	**Age**	**Antigen**	**Dose**	**Antigen**	**Interval[a]**	**Dose**	**Antigen**	**Interval[a]**	**Dose**	**Antigen**	**Interval[a]**	**Dose**
Abbehusen, 2018	IM[o]	2-3 m[g]	LJM17[i]	250 µg	LJM17[x]	28 d[j]	10^8	LJM17[x]	42 d[j]	10^8	NP[l]	NP[l]	NP[l]
Abbehusen, 2018	IM[o]	2-3 m[g]	LJL143[i]	250 µg	LJL143[x]	28 d[j]	10^8	LJL143[x]	42 d[j]	10^8	NP[l]	NP[l]	NP[l]
Aguiar-Soares, 2014	SC[m]	7- 8 m[g]	LB[b] + SGE[c]	600µg[q]	Same[y]	28 d[j]	Same[y]	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Aguiar-Soares, 2014	SC[m]	7-8 m[g]	LB[b] + SGE[c]	600µg[q]	Same[y]	28 d[j]	Same[y]	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Aguiar-Soares, 2014	SC[m]	7-8 m[g]	SGE[c]	UN[f]	Same[y]	28 d[j]	Same[y]	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Alcolea, 2019	IN[p]	12-18 m[g]	pPAL-LACK + pPAL-canIL12-p35 + pPALcanIL12-p40	200µg[r] + 20µg[s] + 20µg[s]	Same[y]	15 d[j]	Same[y]	Same[y]	15 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Borja-Cabrera, 2009	IM[o]	4 m[g]	VR1012-NH36	750 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Bourdoiseau, 2009	SC[m]	UN[f]	LiESAp	100 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Bourdoiseau, 2009	SC[m]	UN[f]	LiESAp	100 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Carcelen, 2009	SC[m]	12-24 m[g]	Q protein	100 µg	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Carcelen, 2009	SC[m]	12-24 m[g]	Q protein	100 µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
De Lima, 2010	SC[m]	UN[f]	FML	1 mL[t]	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
De Lima, 2010	SC[m]	UN[f]	FML	1mL[t]	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Fernandes, 2008	SC[m]	3-9 m[g]	rA2	100 µg[u]	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Fernandes, 2008	SC[m]	3-9 m[g]	rA2	100 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Fernandes, 2008	SC[m]	3-9 m[g]	rA2	100 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Fernandes, 2008	SC[m]	3-9 m[g]	rA2	100 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]

Fiuza, 2015	SC[m]	8 m[g]	FML	1mL[t]	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Fiuza, 2015	SC[m]	8 m[g]	LdCen	10^7	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Gradoni, 2005	SC[m]	6 m[g]	MML	45µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Gradoni, 2005	SC[m]	6 mg	MML	45µg	Same[y]	28 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Lemesre, 2005	SC[m]	12-72 mg	LiESAp	50µg	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Lemesre, 2005	SC[m]	12-72 mg	LiESAp	100 µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Lemesre, 2005	SC[m]	12-72 mg	LiESAp	100 µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Lemesre, 2005	SC[m]	12-72 mg	LiESAp	200 µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Lemesre, 2007	SC[m]	UN[f]	LiESAp	100µg	Same[y]	3-4 w[k]	Same[y]	Same[y]	3-4 w[k]	Same[y]	NP[l]	NP[l]	NP[l]
Martin, 2014	SC[m]	6 m[g]	LiESP/QA-21	100 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Petitdidier, 2016	SC[m]	24-48 m[g]	LaPSA-38S	25 µg	Same[y]	28 d[j]	Same[y]	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Petitdidier, 2016	SC[m]	24-48 m[g]	LaPSA-12S	25 µg	Same[y]	28 d[j]	Same[y]	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Petitdidier, 2019	SC[m]	24-48 m[g]	A17G + A17E + E34PC	25 µg[v] + 25 µg[v] + 10 µg[w]	Same[y]	28 d[j]	Same[y]	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Poot, 2009	SC[m]	6 m[g]	rJPCM5_Q[d]	70µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Poot, 2009	SC[m]	6 m[g]	rJPCM5_Q[d]	70µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Poot, 2009	SC[m]	6 m[g]	rJPCM5_Q[d]	70µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Poot, 2009	SC[m]	6 m[g]	rJPCM5_Q[e]	70µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Poot, 2009	SC[m]	6 m[g]	rJPCM5_Q[e]	70µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Poot, 2009	SC[m]	6 m[g]	rJPCM5_Q[e]	70µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Poot, 2006	SC[m]	6 m[g]	rCPA + rCPB	50 µg + 50 µg	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]

Poot, 2006	SC[m]	6 m[g]	rCPA + rCPB	50 µg + 50 µg	Same[y]	28 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Ramiro, 2003	SC[m]	18-54 m[g]	DNA-LACK	100µg	Same[y]	15 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Ramiro, 2003	SC[m]	18-54 m[g]	DNA-LACK	100µg	rVV-LACK	15 d[j]	10^8 pfu	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Roatt, 2012	SC[m]	UN[f]	LB[b]	600µg	Same[y]	28 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Roatt, 2012	SC[m]	UN[f]	LB[b]	600µg	Same[y]	28 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Roatt, 2012	SC[m]	UN[f]	Saponin	600µg	Same[y]	28 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]
Rodriguez-Cortés, 2007	ID[n]	9 m[g]	pMOK-Kmp11/-TRYP/-LACK/-GP63	200µg[w]	Same[y]	15 d[j]	Same[y]	Same[y]	15 d[j]	Same[y]	Same[y]	Same[y]	15 d[j]
Shahbazi, 2015	SC[m]	6-48 m[g]	pcDNA-A2-CPACPB−CTEGF P (cSLN)	200µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Shahbazi, 2015	SC[m]	6-48 m[g]	pcDNA-A2-CPACPB−CTEGFP (Electroporation)	200µg	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]	NP[l]
Velez, 2020	SC[m]	>6[h] m[g]	LiESP	100 µg	Same[y]	21 d[j]	Same[y]	Same[y]	21 d[j]	Same[y]	NP[l]	NP[l]	NP[l]

[a]Interval: interval between the vaccinations; [b]LB: *L. braziliensis* protein; [c]SGE: Sand fly salivary gland extract*;* [d]rJPCM5_Q: Antigen produced by *E. coli;* [e]rJPCM5_Q: Antigen produced by *Baculovirus;* [f]UN: Uninformed; [g]m: Months; [h]>6: More than 6 months; [i]DNA plasmid; [j]d: Days; [k]w: Weeks; [l]NP: Not performed; [m]SC: subcutaneous; [n]ID: intradermal, [o]IM: intramuscular; [p]IN: Intranasal; [q]Dose of LB protein; [r]Dose of pPAL-LACK; [s]Doses of pPAL-canIL12-p35 and pPALcanIL12-p40; [t]Dose recommended by the vaccine manufacturer; [u]Doses of A17G and A17E; [v]Dose of E34PC; [w]Dose of each plasmid; [x]Expressed by *Canarypoxvirus;* [y]Same antigen and dose as first dose; [x]Promastigotes of *L. infantum*

Supplementary Table S6 – Detailed data on the humoral immune response evaluated through antibodies against the vaccine antigen, produced after vaccination, in trails that performed an experimental challenge and performed this analysis among those selected by this systematic review.

First author, year	Antigen/Adjuvant	ELISA (Antibodies)[a]		Cut off (ELISA)	IFAT[b]	
		Nvac[c] (%)	NC[d] (%)		Nvac[c] (%)	NC[d] (%)
Abbehusen, 2018	LJM17	10/10 (100)	0	0.200	NP[e]	NP[e]
Abbehusen, 2018	LJL143	10/10 (100)	0	0.195	NP[e]	NP[e]
Aguiar-Soares, 2014	LB + SGE/Saponin	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Aguiar-Soares, 2014	LB + SGE	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Aguiar-Soares, 2014	SGE	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Alcolea, 2019	pPAL-LACK	NP[e]	NP[e]	NP[e]	NP[e]	NP[e]
Borja-Cabrera, 2009	VR1012-NH36	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Bourdoiseau, 2009	LiESAp/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Bourdoiseau, 2009	LiESAp/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Carcelen, 2009	Q-Protein	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Carcelen, 2009	Q-Protein	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Fernandes, 2008	rA2/Saponin	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]

Fiuza, 2015	LdCen	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Fiuza, 2015	FML	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Lemesre, 2005	LiESAp/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Lemesre, 2005	LiESAp/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Lemesre, 2005	LiESAp/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Lemesre, 2005	LiESAp/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Martin, 2014	LiESP/QA-2/QA-21	7/10 (70)	0	UE[f]	NP[e]	NP[e]
Petitdidier, 2016	LaPSA-38S/QA-21	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Petitdidier, 2016	LaPSA-12S/QA-21	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Petitdidier, 2019	A17G + A17E + E34PC/QA-21	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]

Poot, 2009	rJPCM5_Q/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Poot, 2009	rJPCM5_Q/ Aluminum hydroxide	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Poot, 2009	rJPCM5_Q/ ISCOMatrix C	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Poot, 2009	rJPCM5_Q/MDP	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Poot, 2006	rCPA + rCPB	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Ramiro, 2003	DNA-LACK	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Ramiro, 2003	DNA-LACK + rVV-LACK	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Roatt, 2012	LBf/ Saponin	NP[e]	NP[e]	NP[e]	NP[e]	NP[e]
Rodriguez-Cortees, 2007	pMOK-Kmp11/-TRYP/-LACK/-GP63	6/6 (100)	2/6 (33.33)	9 EU	NP[e]	NP[e]

Shahbazi, 2015	pcDNA-A2-CPACPB−CTEGFP (cSLN)	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]
Shahbazi, 2015	pcDNA-A2-CPACPB−CTEGFP (Electroporation)	UE[f]	UE[f]	UE[f]	NP[e]	NP[e]

[a]Number of animals that developed a humoral immune response against the vaccine antigen confirmed by ELISA; [b]Number of animals that developed a humoral immune response against the vaccine antigen confirmed by IFAT; cNVac: Vaccinated group; [d]NC: Control group; [e]NP: Not performed; [f]UE: Unable exctract;

Supplementary Table S7 – Detailed data on the humoral immune response evaluated through antibodies against the vaccine antigen, produced after vaccination, in trails that performed an natural challenge and performed this analysis among those selected by this systematic review

First author, year	Antigen/Adjuvant	ELISA (Antibodies)[a]			IFAT[b]	
		Nvac[c] (%)	NC[d] (%)	Cut off (ELISA)	Nvac[c] (%)	NC[d] (%)
De Lima, 2010	FML	UE[g]	UE[g]	UE[g]	NP[e]	NP[e]
Gradoni, 2005[f]	MML + MPL-SE	15/15 (100)	1/15 (6.67)	UE[g]	NP[e]	NP[e]
Gradoni, 2005 [f]	MML + Adjuprime	8/15 (53.33)	1/15 (6.67)	UE[g]	NP[e]	NP[e]
Lemesre, 2007	LiESAp + MDP	21/22 (98.2)	1/33 (2.3)	0.128	NP[e]	NP[e]
Velez, 2020	LiESP + Saponina	NP[e]	NP[e]	NP[e]	NP[e]	NP[e]

[a]Number of animals that developed a humoral immune response against the vaccine antigen confirmed by ELISA; [b]Number of animals that developed a humoral immune response against the vaccine antigen confirmed by IFAT; [c]Vaccinated group; [d]Control group; [e]NP: Not Performed; [f] Results of tests performed 1 year after vaccination; [g]UE: Unable extract;

3. PART TWO: Article 2

This article was written according to the Instructions to the authors of Transboundary and Emerging Diseases journal (Impact Factor: 5.005).

Potential base-antigens for the development of a multiepitope vaccine against Canine Visceral Leishmaniasis: A systematic review

Abstract

Leishmaniasis is caused by protozoa of the genus *Leishmania*, of which dogs are considered primary reservoirs and, therefore, are the focus of many control and prevention measures, especially the use of vaccines. Therefore, the objective of this systematic review was to identify antigens already used in vaccines against Canine Visceral Leishmaniasis, which could have their epitopes used in the construction of a new multiepitope vaccine against the disease and against infection by *L. infantum*. For that, the PRISMA protocol was applied, through the search for articles in online databases, which were selected according to pre-defined criteria by two independent reviewers, who had their differences resolved by a third reviewer, with risk analysis. bias by SYRCLE's RoB tool. The protocol of this review is registered in PROSPERO. This evaluation resulted in the final analysis of 18 studies, highlighting two tested antigens among them. The LACK antigen showed 60% protection against infection by *L. infantum* and 80% protection against LVC and A2, which despite not having shown significant protection rates, demonstrated scientific relevance proven in the literature and, therefore, had their characteristics verified on the TriTrypDB website and its epitopes verified on the IEDB website. Finally, it was concluded that the two antigens can be used in a new multiepitope vaccine, however, it is necessary to define the most immunogenic epitopes that are capable of stimulating good levels of protection of animals against CVL and infection by the protozoan. In addition to these, other antigens that stimulated good cellular and humoral immune responses can also be considered to compose the new vaccine, provided that their efficacy rates are better determined.

Keywords: Leishmaniasis, Dogs, Bioinformatics, Protein, Antigen, Epitope

1- Introduction

Canine visceral leishmaniasis (CVL) is a disease caused by protozoa of the genus *Leishmania*, most commonly by the species *L. infantum*, usually transmitted by the female sand fly *Lutzomyia longipalpis*. Animals susceptible to the disease commonly develop clinical signs characterized by changes in the skin and annexes, such as onychogryphosis, alopecia, ulcers and dermatitis, ocular changes, such as keratoconjunctivitis and uveitis, weight loss, apathy, hepatosplenomegaly, renal failure, in addition to presenting various alterations in biochemical tests (Picón et al., 2020). Some animals show some resistance to the parasite and, therefore, do not show significant clinical changes (Shokri et al., 2017).

CVL is considered a public health problem since the number of cases of the disease in dogs is directly related to the number of human cases of Leishmaniasis, because the parasite, residing in the skin of animals, is also transmitted in a vector way to people (Marcondes & Day, 2019). CVL can be prevented by controlling the vector population, applying insecticides in homes, applying protective screens on doors and windows, and cleaning environments conducive to the accumulation of organic matter (Gonçalves et al., 2019). Measures aimed at the canine population can also be applied, such as the use of repellent collars, preventing animals from circulating in environments where sandflies can inhabit, especially at twilight, and vaccination (Reguera et al., 2016). The latter is considered an individual protection measure because the vaccines available on the market still protect against the disease and not against the infection of animals. However, it can be used as an important public health measure if it achieves desired levels of protection against infection, reducing the circulation of the parasite in the canine population through the formation of a satisfactory collective immunity, reducing the number of infected vectors and humans. For this reason, it is necessary to develop a new vaccine antigen that protects against disease and infection, stimulating immune mechanisms linked to animal resistance, in addition to meeting ideal characteristics such as fewer applications and lower costs.

To develop new immunogens that protect against CVL, reverse vaccinology can be used, identifying, through bioinformatics tools, potential antigens that can be used in vaccines (Silva, 2017). Reverse vaccinology was initially used in the 1990s to predict antigens for a vaccine against the B strains of *Neisseria meningitidis* (meningococci) (Moxon et al., 2019). Since then, many advances have been made, with significant improvements in the quality and accuracy of the tools used for antigen selection (Donati

& Rappuoli, 2013). The creation of databases that gather information about antigens stands out among these advances. TriTrypDB, for example, is a database that was first released in 2009, in a collaboration between the EuPathDB group at the Universities of Pennsylvania and Georgia, the GeneDB group at the Wellcome Trust Sanger Institute, and researchers at Seattle Biomedical research Institute, which gathers a lot of information on parasites of the order Kinetoplastida, being relevant in the various researches with vaccines against *Leishmania* species and other trypanosomatids (Aslett et al., 2010). Another relevant database is the Immune epitope Database (IEDB). The IEDB is a site where a lot of information can be found on epitopes related to various species of infectious agents and hosts, gathering data from tables, texts, and figures from the literature so that the prediction of epitopes is facilitated, also indicating the best predictors for these molecules (Vita et al., 2019).

TriTrypDB and IEDB databases, as well as other specialized software, can be used to facilitate the construction of a new vaccine antigen, as they can analyze the structure and provide information such as molecular weight, isoelectric point, genetic sequence, and even behavior prediction. Of the immune system of individuals when in contact with this protein (Nogueira, 2019). All of this can lead to less waste of resources because in silico analysis, often free of charge, can be performed before laboratory tests, which, when better targeted, generate savings in inputs, time, and consequently, money. In addition to bioinformatics tools, previously developed research can also be used to select promising antigens. This was carried out in this systematic review, which aimed to identify antigenic components previously used in vaccines against Canine Visceral Leishmaniasis, which could have their epitopes used in the construction of a new multiepitope vaccine against the disease and against infection by *L. infantum*.

2- Material and methods

The guidelines of PRISMA statement (Preferred Reported Items for Systematic Reviews and Meta-Analysis) were adopted in this review (Supplementary Table S1).

Details of the protocol for this systematic review were registered on PROSPERO and can be accessed in:

https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42021264345

2.1- Search strategy and selection of the studies

The searches for the studies that make up this review began on July 12, 2021 and were carried out in the Pubmed, Web of Science, Scopus, Cochrane, Scielo, CABI, Science direct and BVS databases, with terms searched in the title, abstract and full text. The PICOT (population, intervention, comparison, outcome and time) used for searches is described in Supplementary Table S2.

Initially, the studies found in the databases were selected based on the title by two reviewers (JAMP and TFM), who paid attention to the words "Vaccine" or "Leishmaniasis" to carry out the selection. Subsequently, the selection of studies by abstract was carried out, also by two reviewers (JAMP and TFM). Abstracts that did not contain information on leishmaniasis and vaccines were excluded. The full texts were then analyzed by two reviewers (JAMP and TFM) and included or excluded based on predetermined criteria. Disagreements between the two reviewers were resolved by a third reviewer (APP).

2.2- Inclusion and exclusion criteria

The studies included in the review were those that fit the following criteria: (i) published in all countries, (ii) published in all years, (iii) that talked about vaccines used for prophylaxis, (iv) against Canine Visceral Leishmaniasis, (v) in dogs, (vi) *L. infantum* species. Studies in languages other than English, Spanish, or Portuguese that tested vaccines composed of the attenuated or inactivated protozoan and excreted/secreted Leishmania antigens and that met the exclusion criteria detailed in Supplementary Table S3 were excluded. This step was performed by two reviewers (JAMP and TFM).

2.3- Risk of bias analysis by SYRCLE's RoB Tool

After the inclusion of the studies by pre-established criteria, the analysis of the risk of bias of the studies was carried out through a specific protocol, called SYRCLE's RoB tool (Hoojimans et al., 2014). This protocol consists of evaluating five different types of bias: (i) selection bias, (ii) performance bias, (iii) detection bias, (iv) attrition bias, (v) reporting bias, and (vi) other biases. These five types of bias are divided into ten questions or domains that must be answered with "Yes", "Unclear" or "No", which respectively mean low, uncertain, or high risks of bias.

2.4- Type of studies

Only original studies were included. Cohort, case-control, cross-sectional, case series, case reports and reviews were excluded.

2.5- Data extraction

Data were extracted by one of the reviewers (JAMP) and verified by one reviewer (TFM). First author, year of publication, the country where the study was carried out, type of vaccine antigen tested, characteristics of the groups evaluated, such as age, sex, number of animals total and per group, breed, dose, number of vaccinations, the time between administration of doses, type of adjuvant, and route of administration. In addition, it extracted how many animals there were in the control groups and what was applied to them, the period, dose and route of the experimental challenge, and the period of exposure to the parasite, when it came to studies that performed natural challenge, the diagnostic methods used to confirm infection, as well as tests performed to verify the immunogenicity of the antigen, were the data extracted from each study for further analysis.

2.6- Data analysis

Chi-Square or Fisher's exact tests were used for the calculation of Relative Risk (RR), considering a confidence interval (CI) of 95% ($p < 0.05$), in the GraphPad Prism Software (version 8.0). To calculate vaccine protection rates, the "1-RR" formula was used, using as data the number of vaccinated and control dogs that showed clinical signs of Canine Visceral Leishmaniasis and the number of vaccinated dogs and the control group that showed positivity in the diagnostic tests. Studies that did not assess infection by diagnostic methods or did not assess clinical signs were excluded from this analysis.

2.7- Identification of the genetic sequences and epitopes that make up the antigens

Protein vaccine antigens with minimum rates of protection against disease or infection of 20%, had information such as identification number (ID), isoelectric point, molecular weight, and length extracted from the TriTrypDB database: Kinetoplastid genomics Resource. Information about the epitopes that compose them was taken from Immune epitope Database and Analysis Resource, (IEDB). Proteins without significant protection rates, but with proven relevance in the literature, were also searched.

3- Results

3.1 – Study selection and characteristics

Initially, 14.959 were found studies and 3.705 duplicates were detected among them. 11.254 were included in the selection by titles and at the end of the entire process, 59 studies were analyzed by eligibility criteria (Figure 1). Of these, 18 were eligible for the final analyzes (Table 1) and were classified according to the risk of bias by SYRCLE's RoB Tool (Figure 2). No study showed a high risk of bias in any of the domains (Figure 3). All these studies were carried out between 2003 and 2019. Articles excluded after the selection stage by eligibility criteria and the reasons why they were not included in this review are in Supplementary Table S4. Studies that performed more than one experiment, analyzed different antigens or the same antigen at different times, compared to more than one control group or with different challenge characteristics, such as time or dose, were defined as trials.

Figure 1 – PRISMA flowchart used in the selection of the studies for this systematic review.

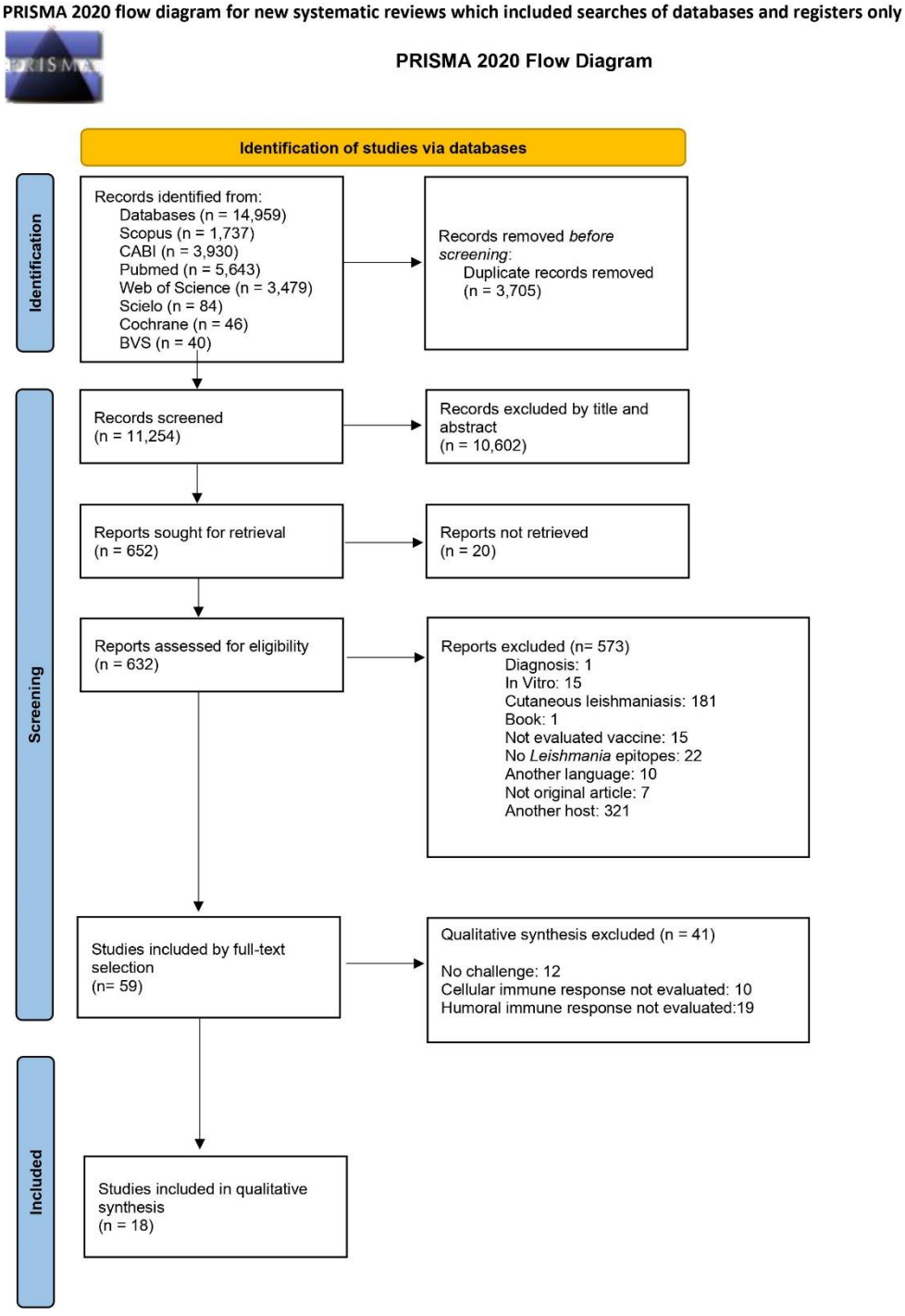


Table 1 – General characteristics of studies includes in the systematic review

First author, year	Country	Type of study	Total number of animals	Additional vaccines[d]	Dog breeds	Sex	Age
Abeijon, 2016[a]	United States	Intervention[b]	20	UN[e]	Beagle	Males	6 m[l]
Alcolea, 2019	Spain	Intervention[b]	10	UN[e]	Beagle	Both[k]	12-18 m[l]
Borja-Cabrera, 2009[a]	Brazil	Intervention[b]	19	Group 1[f]	UB[j]	Both[k]	4 m[l]
Borja-Cabrera, 2008[a]	Brazil	NI[c]	1138	Group 3[h]	UB[j]	Both[k]	UN[g]
Carcelen, 2009	Spain	Intervention[b]	21	UN[e]	Beagle	Both[k]	12-24 m[l]
Fernandes, 2008	Brazil	Intervention[b]	21	Group 1[f]	Beagle	Both[k]	3-9 m[l]
Goto, 2007	Spain	Intervention[b]	6	UN[e]	UN[e]	Both[k]	8-36 m[l]
Petitdidier, 2016	France	Intervention[b]	19	Routine vaccines	Beagle	Both[k]	24-48 m[l]
Petitdidier, 2019	France	Intervention[b]	15	Routine vaccines	Beagle	Both[k]	24-48 m[l]
Poot, 2009	Germany	Intervention[b]	44	UN[e]	Beagle	Both[k]	6 m[l]
Poot, 2006	France	Intervention[b]	15	UN[e]	Beagle	Males	6 m[l]
Ramiro, 2003	Spain	Intervention[b]	20	Group 2[g]	Beagle	Both[k]	18-54 m[l]
Ramos, 2008	Spain	Intervention[b]	16	Group 2[g]	Beagle	Both[k]	12-18 m[l]
Ramos, 2009	Spain	Intervention[b]	16	Group 2[g]	Beagle	Both[k]	12-18 m[l]
Roatt, 2012	Brazil	Intervention[b]	20	Group 1[f]	UB[j]	Both[k]	UN[g]
Rodriguez-Cortés, 2007	Spain	Intervention[b]	12	Group 3[h]	Beagle	Females	9 m[l]
Shahbazi, 2015	Iran	Intervention[b]	30	Group 1[f]	UB[j]	Both[k]	6-48 m[l]
M. Shahbazi, 2015	Iran	Intervention[b]	30	Group 4[i]	UB[j]	Both[k]	6-48 m[l]
R. Sima, 2005	Iran	Intervention[b]	15	Group 4[i]	UB[j]	Both[k]	12 m[l]

[a]Only some data were taken from this study, which fit the quality criteria; [b]Studies that performed experimental infection in dogs. [c]NI: Natural Infection; [d]Other vaccines given to animals other than CVL; [e]UN: Uninformed; [f]Group 1: Vaccines group composed of Rabies, distemper, hepatitis, adenovirus, leptospirosis

Figure 2 – Risk of bias summary: Authors' judgments of each risk of bias item for each included study, based on SYRCLE's RoB tool.

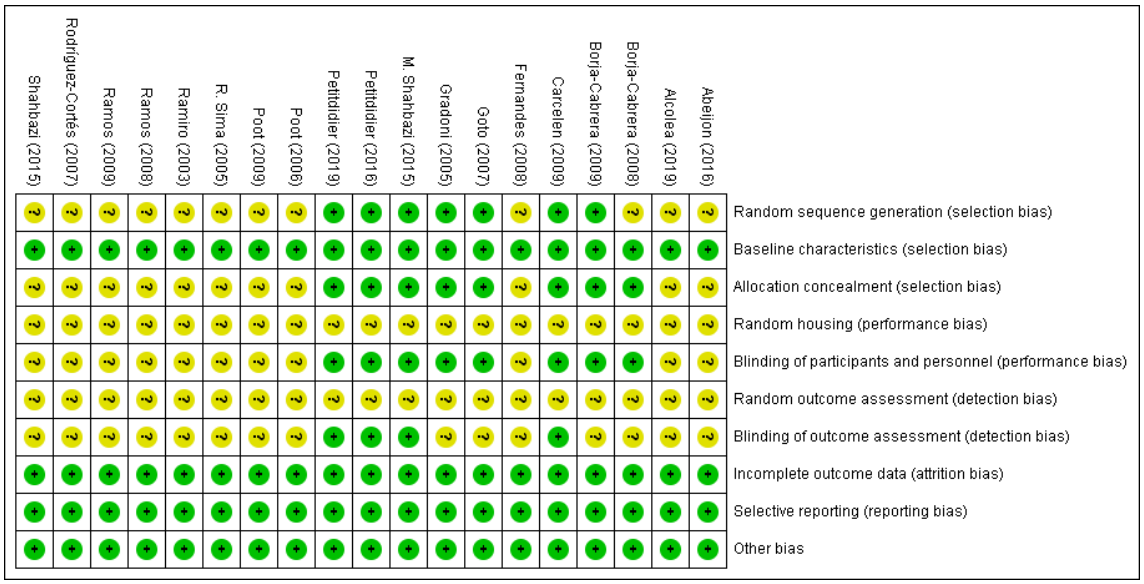
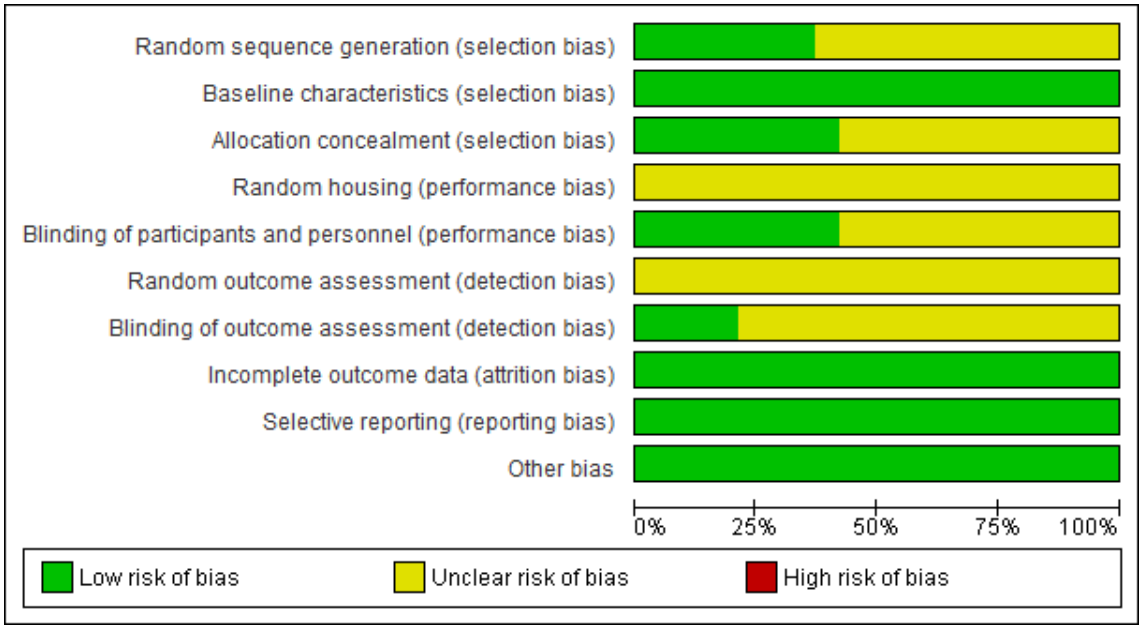


Figure 3 – Risk of bias graph: Author's judgments about each risk of bias item based on in SYRCLE's RoB tool, presented as percentages across all included studies.



3.2- Basic information about trials and vaccines

Of the selected studies, only one performed a natural challenge, in which the vaccinated animals and the control group are in their natural environments, so 94.4 % (17/18) of the studies performed experimental infections to verify vaccine efficacy. The breed most used for the tests was the beagle breed, and the only study that performed a natural challenge did not use defined breed animals. The groups consisted mostly of dogs of both sexes, only 11.1% (2/18) of the studies used males, and only one of the studies used females (Rodriguez-Cortés et al., 2007). The minimum age adopted was 3 months and the maximum 48 months (Table 1). The antigens tested in the 47 trials are described in Table 2 and were mostly applied subcutaneously (61.7%), at the frequencies of: one vaccination (2.1%), two vaccinations (55.3%), three vaccinations (40.5%) or four vaccinations (2.1%) (Supplementary Table S5).

Table 2 – Vaccination and challenge data from trials selected for systematic review.

First author, year	Vaccine's generation	N Vac[c]	N C[d]	Control Group	Vaccination				Challenge			
					Antigen/Adjuvant	Age (Months)	Route	Type of Challenge	Strain	Dose[r]	Route	Time (days)
Abeijon, 2016	2a[a]	10	10	P[e]	Li-ntf2/ BpMPLA-SE	6	SC[j]	E[n]	LIP[p]	10^7	IV[s]	30 d
Alcolea, 2019	3a[b]	5	5	P[e]	pPAL-LACK	12-18	IN[m]	E[n]	LIP[p]	10^8	UN[t]	60 d
Borja-Cabrera, 2009	3a[b]	6	13	P[e]	VR1012-NH36	4	IM[l]	E[n]	LIA[q]	$7x10^8$	UN[t]	67 d[u]
Borja-Cabrera, 2008	2a[a]	550	588	P[e]	FML	UN	SC[j]	NI[o]	NI[o]	NI[o]	NI[o]	210 d
Borja-Cabrera, 2008	2a[a]	550	588	P[e]	FML	UN	SC[j]	NI[o]	NI[o]	NI[o]	NI[o]	365 d
Carcelen, 2009	2a[a]	7	7	P[e]	Q-Protein	12-24	SC[j]	E[n]	LIP[p]	10^5	IV[s]	60 d
Carcelen, 2009	2a[a]	7	7	P[e]	Q-Protein	12-24	SC[j]	E[n]	LIP[p]	10^5	IV[s]	60 d
Fernandes, 2008	2a[a]	14	7	P[e]	rA2/Saponin	3-9	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	28 d
Fernandes, 2008	2a[a]	14	7	P[e]	rA2/Saponin	3-9	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	28 d
Fernandes, 2008	2a[a]	14	4	P[e]	rA2/Saponin	3-9	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	28 d
Fernandes, 2008	2a[a]	14	3	A[f]	rA2/Saponin	3-9	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	28 d
Goto, 2007	2a[a]	8	4	A[f]	HASPB1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	A[f]	H1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	A[f]	HASPB1 + H1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	A[f]	MML/MPL-SE	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	A[f]	HASPB1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	A[f]	H1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	A[f]	HASPB1 + H1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	A[f]	MML/MPL-SE	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	P[e]	HASPB1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	P[e]	H1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	P[e]	HASPB1 + H1/Montanide	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Goto, 2007	2a[a]	8	4	P[e]	MML/MPL-SE	8-36	ID[k]	E[n]	LIP[p]	10^8	IV[s]	45 d
Petitdidier, 2016	2a[a]	9	5	P[e]	LaPSA-38S/QA-21	24-48	SC[j]	E[n]	LIP[p]	10^8	IV[s]	60 d

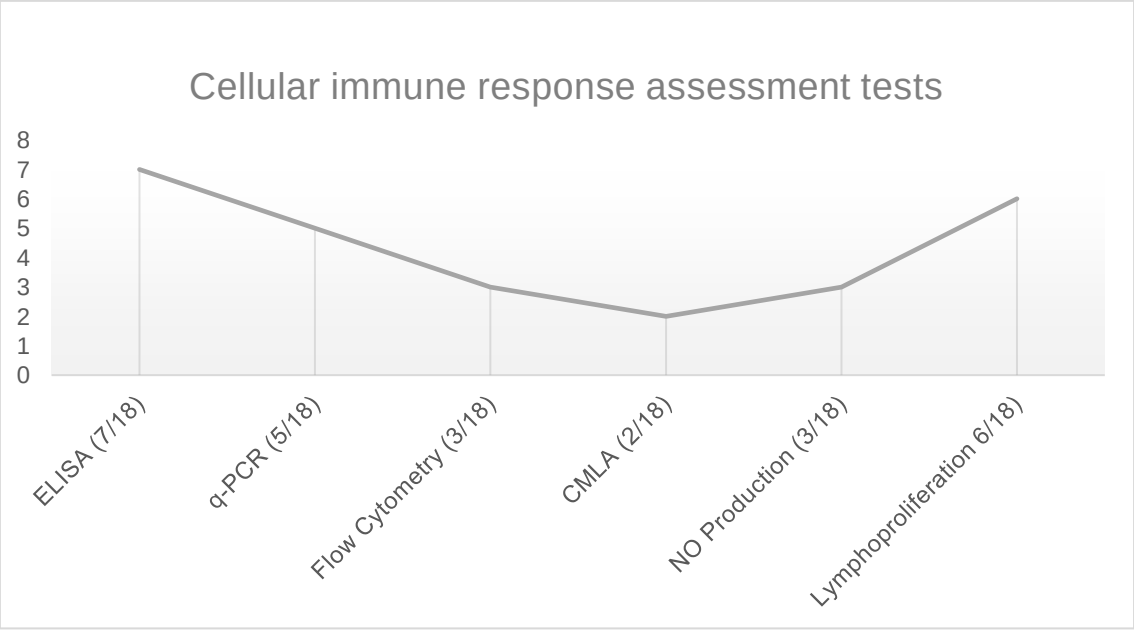
Petitdidier, 2016	2a[a]	5	5	P[e]	LaPSA-12S/QA-21	24-48	SC[j]	E[n]	LIP[p]	10^8	IV[s]	60 d
Petitdidier, 2019	2a[a]	10	5	P[e]	A17G + A17E + E34PC/QA-21	24-48	SC[j]	E[n]	LIP[p]	10^8	IV[s]	120 d
Poot, 2009	2a[a]	7	7	P[e]	rJPCM5_Q[h]/ MDP	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	28 d
Poot, 2009	2a[a]	7	7	P[e]	rJPCM5_Q[h]/ Aluminum hydroxide	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	28 d
Poot, 2009	2a[a]	7	7	P[e]	rJPCM5_Q[h]/ ISCOMatrix C	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	28 d
Poot, 2009	2a[a]	5	1	P[e]	rJPCM5_Q[i]/ MDP	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	21 d
Poot, 2009	2a[a]	5	1	P[e]	rJPCM5_Q[i]/ Aluminum hydroxide	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	21 d
Poot, 2009	2a[a]	5	1	P[e]	rJPCM5_Q[i]/ ISCOMatrix C	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	21 d
Poot, 2006	2a[a]	5	5	P[e]	rCPA + rCPB/ rIL-12	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	21 d
Poot, 2006	2a[a]	5	5	P[e]	rCPA + rCPB/ rIL-12 + QuilA	6	SC[j]	E[n]	LIP[p]	$5x0^7$	IV[s]	21 d
Ramiro, 2003	3a[b]	5	5	P[e]	DNA-LACK	18-54	SC[j]	E[n]	LIP[p]	10^8	IV[s]	0 d
Ramiro, 2003	3a[b]	5	5	P[e]	DNA-LACK + rVV-LACK	18-54	SC[j]	E[n]	LIP[p]	10^8	IV[s]	0 d
Ramos, 2008	3a[b]	4	5	P[e]	DNA-LACK + rVV-LACK	12-18	SC[j]	E[n]	LIP[p]	10^8	IV[s]	15 d
Ramos, 2008	3a[b]	4	5	P[e]	DNA-LACK + MVA-LACK	12-18	SC[j]	E[n]	LIP[p]	10^8	IV[s]	15 d
Ramos, 2009	3a[b]	8	5	P[e]	pORT-LACK + MVA – LACK	12-18	SC[j]	E[n]	LIP[p]	10^8	IV[s]	15 d
Rodriguez-Cortés, 2007	3a[b]	6	6	P[e]	pMOK-Kmp11/-TRYP/-LACK/-GP63	9	ID[k]	E[n]	LIP[p]	$5x0^7$	IV[s]	30 d
Shahbazi, 2015	3a[b]	10	10	P[e]	pcDNA-A2-CPACPB−CTEGF P (cSLN)	6-48	SC[j]	E[n]	LIP[p]	$4x0^7$	IV[s]	42 d
Shahbazi, 2015	3a[b]	10	10	P[e]	pcDNA-A2-CPACPB−CTEGFP (Electroporation)	6-48	SC[j]	E[n]	LIP[p]	$4x0^7$	IV[s]	42 d
M. Shahbazi, 2015	2a[a]	10	10	P[e]	*L. tarentolae* A2-CPA-CPB-CTE EGFP	6-48	SC[j]	E[n]	LIP[p]	$4x0^7$	IV[s]	21 d
M. Shahbazi, 2015	2a[a]	10	10	V[g]	*L. tarentolae* A2-CPA-CPB-CTE EGFP	6-48	SC[j]	E[n]	LIP[p]	$4x0^7$	IV[s]	21 d
R. Sima, 2005	3a[b]	10	2	P[e]	pCB6-cpa + pCB6-cpb; rCPs + ODN CPG	12	IM[l]	E[n]	LIP[p]	$5x10^6$	IV[s]	30 d
R. Sima, 2005	3a[b]	10	2	V[g]	pCB6-cpa + pCB6-cpb; rCPs + ODN CPG	12	IM[l]	E[n]	LIP[p]	$5x10^6$	IV[s]	30 d

[a]2a: Second Generation; [b]3a: Third Generation; [c]N Vac: number of vaccinated animals; [d]N C: number of control animals; [ex]P: Placebo; [f]A: Adjuvant; [g]V: Empty vector; [h]rJPCM5_Q: Antigen produced by *E. coli;* [i]rJPCM5_Q: Antigen produced by *Baculovirus;* [j]SC: subcutaneous; [k]ID: intradermal, [l]IM: intramuscular; [m]IN: Intranasal; [n]E: Experimental challenge; [o]NC: Natural challenge; [p]LIP: *L. infantum* promastigotes; [q]LIA: *L. infantum* amastigotes; [r]Dose: Unity = Parasites; [s]IV: Intravenous; [t]UN: Uninformed; [u]d: Days after first dose

3.3 – Methods to confirm immunogenicity of antigens

The ability to stimulate an immune response in the hosts was evaluated through tests that analyzed cellular and humoral immune responses. To confirm the production of antibodies, all studies used the Enzyme-linked immunosorbent assay (ELISA), however, most of these results in the studies were summarized in graphs, and therefore, it was not possible to extract them (Supplementary Table S6). However, two studies presented clear numerical results. The first (Borja-Cabrera et al., 2008), demonstrated that the FML antigen was able to stimulate a humoral immune response in 423 of the 432 vaccinated dogs, representing 98%, however, the animals in the control group (15.6% - 98/588) also showed specific antibodies against the antigen. The second study (Rodriguez-Cortés et al., 2007) also demonstrated the stimulation of humoral immune response against the pMOK-Kmp11/-TRYP/-LACK/-GP63 antigen in 100% (6/6) of vaccinated dogs and 33.33 % (2/6) of control dogs. In questions of the cellular immune response, ELISA tests were used to detect cytokines in the serum of the animals and cell culture supernatant, as well as cell types in these cultures, q-PCR, also for the detection of cytokines in cell culture, cytometry of flow, canine macrophage Leishmanicidal Assay (CMLA), NO production by cells and lymphoproliferation. The number of studies that used each test is shown in Figure 4.

Figure 4 – Percentage of tests used by studies that performed experimental and natural infection to evaluate cellular immune response against vaccinal antigen.



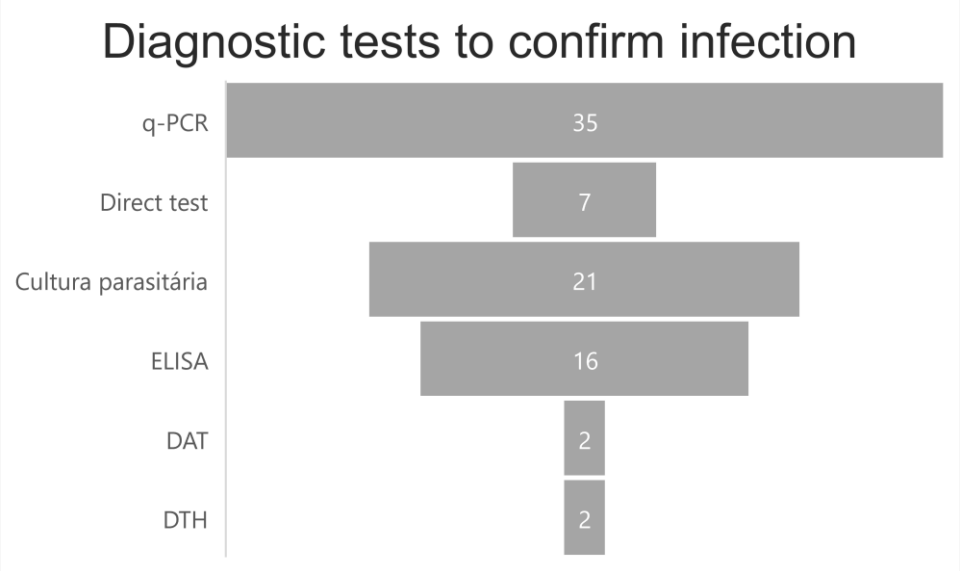
3.4- Challenge strains, dose and route of exposure

All dogs from all trials that underwent experimental infection (45) were challenged with *L. infantum* promastigotes. At doses of 10^8 parasites, in 46.7% (21/45) of trials, $5x10^7$ parasites in 28.9% (13/45) of trials, $4x10^7$ parasites in 8.5% (4/45) of trials. The rest of the doses used are shown in Table 2, as well as the challenge time, which ranged from 0 to 120 days. The route used for the challenge in 95.6% (43/45) of the trials was intravenous, and two trials (Alcolea et al., 2019; Borja-Cabrera et al., 2009) did not inform which route was used. The trials that performed a natural challenge, evaluated the dogs in the periods of 210 and 365 days of exposure after the vaccinations.

3.5- Diagnostic methods to confirm infection and evaluation of clinical signs

The diagnostic methods used to confirm *L. infantum* infection are shown in Figure 5. The most used was q-PCR, performed by 35 of the trials, followed by parasite culture, which was performed by 21 of the trials. As for clinical signs, the main ones related to Canine Visceral Leishmaniasis were evaluated, namely Alopecia, ulcers, exfoliative dermatitis, onychogryphosis, weight loss, eye lesions such as uveitis, conjunctivitis, and keratoconjunctivitis and lymphadenopathy. This evaluation was used as a way of differentiating symptomatic from asymptomatic dogs, among the vaccinated animals and the control group, in 46.8% (22/47) trials.

Figure 5 – Diagnostic methods to confirm infection by *L. infantum*



3.6- Assessment of protection against disease and infection by L. infantum

The protection rates against infection and disease were calculated considering, respectively, animals with proven infection in at least one diagnostic test among those performed by the studies and dogs that presented clinical signs related to Canine Visceral Leishmaniasis. The vaccine composed of the LACK antigen, administered to animals in two different forms in different vaccinations, presented a protection rate of 80% (RR = 0.2, 95% CI: 0.03 – 0.66) against the disease (Ramiro et al., 2003). The same study showed a 60% (RR = 0.4, 95% CI: 0.11 – 0.93) rate of protection against *L. infantum* infection. The other antigens tested did not show significant rates of protection against the disease (Table 3) or infection (Table 4).

Table 3 – Efficacy of the vaccine antigen against the development of CVL in vaccinated and unvaccinated animals in studies that performed experimental challenge.

First author, year	Antigen/Adjuvant	Control Group	Challenge dose	Asymptomatic animals[c]		Symptomatic animals[d]		Relative Risk	[e]CI (95%)	Vaccine Efficacy
				NVac[a](%)	NC[b](%)	NVac[a](%)	NC[b](%)			(1 - RR[f])
Alcolea, 2019	pPAL-LACK	Placebo	10^8	1/5 (20)	1/5 (20)	4/5 (80)	4/5 (80)	1	0.44-2.25	0
Carcelen, 2009	Q-Protein	Placebo	10^5	4/7 (57.15)	1/7 (14.3)	3/7 (42.9)	6/7 (85.7)	0.5	0.17-1.13	0.5
Carcelen, 2009	Q-Protein	Placebo	10^5	2/7 (28.57)	1/7 (14.3)	5/7 (71.42)	6/7 (85.7)	0.833	0.40-1.59	0.16
Fernandes, 2008	rA2/Saponin	Placebo	5x10^7	5/7 (71.42)	2/7 (28.57)	2/7 (28.,57)	5/7 (71.42)	0.4	0.10-1.20	0.6
Goto, 2007	HASPB1	Montanide	10^8	4/8 (50)	1/4 (25)	4/8 (50)	3/4 (75)	0.6667	0.2627 - 1.924	0.3333
Goto, 2007	HASPB1	MPL-SE	10^8	4/8 (50)	0	4/8 (50)	4/4 (100)	0.5	0.2152 - 1.140	0.5
Goto, 2007	HASPB1	Placebo	10^8	4/8 (50)	2/8 (25)	4/8 (50)	6/8 (75)	0.6667	0.2711 - 1.470	0.3333
Goto, 2007	H1	Montanide	10^8	5/8 (62.5)	1/4 (25)	3/8 (37.5)	3/4 (75)	0.5	0.1683 - 1.573	0.5
Goto, 2007	H1	MPL-SE	10^8	5/8 (62.5)	0	3/8 (37.5)	4/4 (100)	0.375	0.1368 - 0.9352	0.625
Goto, 2007	H1	Placebo	10^8	5/8 (62.5)	2/8 (25)	3/8 (37.5)	6/8 (75)	0.5	0.1736 - 1.226	0.5
Goto, 2007	HASPB1 + H1	Montanide	10^8	4/8 (50)	1/4 (25)	4/8 (50)	3/4 (75)	0.6667	0.2627 - 1.924	0.3333
Goto, 2007	HASPB1 + H1	MPL-SE	10^8	4/8 (50)	0	4/8 (50)	4/4 (100)	0.5	0.2152 - 1.140	0.5
Goto, 2007	HASPB1 + H1	Placebo	10^8	4/8 (50)	2/8 (25)	4/8 (50)	6/8 (75)	0.6667	0.2711 - 1.470	0.3333
Goto, 2007	MML	Montanide	10^8	2/7 (28.5)	1/4 (25)	5/7 (71.4)	3/4 (75)	0.9524	0.4363 - 2.541	0.0476

Goto, 2007	MML	MPL-SE	10^8	2/7 (28.5)	0	5/7 (71.4)	4/4 (100)	0.7143	0.3589 - 1.500	0.2857
Goto, 2007	MML	Placebo	10^8	2/7 (28.5)	2/8 (25)	5/7 (71.4)	6/8 (75)	0.9524	0.4498 - 1.900	0.0476
Ramiro, 2003	DNA-LACK	Placebo	10^8	0	0	5/5 (100)	5/5 (100)	1	0.56- 1.76	0
Ramiro, 2003	DNA-LACK + rVV-LACK	Placebo	10^8	4/5 (80)	0	1/5 (20)	5/5 (100)	0.2	0.03- 0.66	0.8
Ramos, 2009	pORT-LACK + MVA-LACK	Placebo	10^8	5/8 (62.5)	1/5 (20)	3/8 (37.5)	4/5 (80)	0.4688	0.1622 - 1.291	0.5312
Rodriguez-Cortés, 2007	pMOK-Kmp11/-TRYP/-LACK/-GP63		5x10^7	4/6 (66.67)	2/6 (33.33)	2/6 (33.33)	4/6 (66.67)	0.5	0.13-1.58	0.5
M. Shahbazi, 2015	*L. tarentolae* A2-CPA-CPB-CTE EGFP	Placebo	4x10^7	7/10 (70)	4/10 (40)	3/10 (30)	6/10 (60)	0.5	0.1657 - 1.345	0.5
M. Shahbazi, 2015	*L. tarentolae* A2-CPA-CPB-CTE EGFP	Vetor	4x10^7	7/10 (70)	7/10 (70)	3/10 (30)	3/10 (30)	1	0.2782 - 3.595	0

[a]N Vac: Number of vaccinated animals; [b]N C: Number of control animals; [c]Animals without clinical signs; [d]Animals with clinical signs; [e]Confidence interval; [f]RR: Relative Risk.

Table 4 – Efficacy of the vaccine antigen against *L. infantum* infection in vaccinated and unvaccinated animals in studies that performed experimental challenge**.**

First author, year	Antigen/Adjuvant	Control Group	Challenge dose	Positive Animals[c]		Negative Animals[d]		Relative Risk	[e]CI (95%)	Vaccine Efficacy
				NVac[a](%)	NC[b](%)	NVac[a](%)	NC[b](%)			(1 - RR[f])
Alcolea, 2019	pPAL-LACK	Placebo	10^8	5/5 (100)	5/5(100)	0	0	1	0.56- 1.76	0
Carcelen, 2009	Q-Protein	Placebo	10^5	5/7 (71.4)	7/7 (100)	2/7 (28.57)	0	0.7143	0.35-1.18	0.2857
Carcelen, 2009	Q-Protein	Placebo	10^5	6/7 (85.71)	7/7 (100)	1/7 (14.28)	0	0.8571	0.48-1.36	0.1429
Fernandes, 2008	rA2/Saponin	Placebo	5x10^7	4/7 (57.15)	7/7 (100)	3/7 (42.85)	0	0.5714	0.25-1.0	0.4286
Goto, 2007	HASPB1	Montanide	10^8	6/8 (75)	4/4 (100)	2/8 (25)	0	0.75	0.409 - 1.550	0.25
Goto, 2007	HASPB1	MPL-SE	10^8	6/8 (75)	3/4 (75)	2/8 (25)	1/4 (25)	1	0.4961 - 2.626	0
Goto, 2007	HASPB1	Placebo	10^8	6/8 (75)	5/8 (62.5)	2/8 (25)	3/8 (37.5)	I[g]	I[g]	I[g]
Goto, 2007	H1	Montanide	10^8	5/8 (62.5)	4/4 (100)	3/8 (37.5)	0	0.625	0.3057 - 1.345	0.375
Goto, 2007	H1	MPL-SE	10^8	5/8 (62.5)	3/4 (75)	3/8 (37.5)	1/4 (25)	0.8333	0.3713 - 2.275	0.1667
Goto, 2007	H1	Placebo	10^8	5/8 (62.5)	5/8 (62.5)	3/8 (37.5)	3/8 (37.5)	1	0.4387 - 2.279	0
Goto, 2007	HASPB1 + H1	Montanide	10^8	6/8 (75)	4/4 (100)	2/8 (25)	0	0.75	0.4093 - 1.550	0.25
Goto, 2007	HASPB1 + H1	MPL-SE	10^8	6/8 (75)	3/4 (75)	2/8 (25)	1/4 (25)	1	0.4961 - 2.626	0
Goto, 2007	HASPB1 + H1	Placebo	10^8	6/8 (75)	5/8 (62.5)	2/8 (25)	3/8 (37.5)	I[g]	I[g]	I[g]
Goto, 2007	MML	Montanide	10^8	5/7 (71.4)	4/4 (100)	2/7 (28.6)	0	0.7143	0.3589 - 1.500	0.2857
Goto, 2007	MML	MPL-SE	10^8	5/7 (71.4)	3/4 (75)	2/7 (28.6)	1/4 (25)	0.9524	0.4363 - 2.541	0.0476
Goto, 2007	MML	Placebo	10^8	5/7 (71.4)	5/8 (62.5)	2/7 (28.6)	3/8 (37.5)	I[g]	I[g]	I[g]

Ramiro, 2003	DNA-LACK	Placebo	10^8	5/5 (100)	5/5 (100)	0	0	1	0.56-1.76	0
Ramiro, 2003	DNA-LACK + rVV-LACK	Placebo	10^8	2/5 (40)	5/5 (100)	3/5 (60)	0	0.4	0.11- 0.93	0.6
Ramos, 2009	pORT-LACK + MVA-LACK	Placebo	10^8	6/8 (75)	8/8 (100)	2/8 (25)	0	0.4688	0.1622 - 1.291	0.5312
Rodriguez-Cortés, 2007	pMOK-Kmp11/-TRYP/-LACK/-GP63		5x10^7	6/6 (100)	2/6 (33.33)	0	4/6 (66.67)	I[g]	I[g]	I[g]
M. Shahbazi, 2015	*L. tarentolae* A2-CPA-CPB-CTE EGFP	Placebo	4x10^7	9/10 (90)	9/10 (90)	1/10 (10)	1/10 (10)	1	0.6486 - 1.542	0
M. Shahbazi, 2015	*L. tarentolae* A2-CPA-CPB-CTE EGFP	Vetor	4x10^7	9/10 (90)	8/10 (80)	1/10 (10)	2/10 (20)	I[g]	I[g]	I[g]

[a]N Vac: Number of vaccinated animals; [b]N C: Number of control animals; [c]Positive animals in at least one diagnostic test performed by the study; [d]Negative animals in all diagnostic tests performed by the study; [e]Confidence interval; [f]RR: Relative Risk; [g]I:lmpossible to calculate

3.7- Antigen information

The LACK antigen or Receptor for activated C kinase 1, from *L. infantum* evaluated by several studies selected in this systematic review, is registered in TriTrypDB under identification number (ID) LINF_280034700. This antigen, named RACK1, is located on chromosome 28, at location LinJ.28:1,070,378.. 1,071,316(-), of the species *L. infantum*, strain JPCM5, considered the reference strain of the species. Its isoelectric point is 6.49, with a molecular weight of 34373kDa and a length of 312. There is no information about its epitopes registered in the IEDB.

The A2 antigen, despite not showing significant protection rates according to the calculations made in this review, showed promise when researched in the literature and, therefore, may be composed of epitopes that could be used in the construction of a new vaccine against CVL and, therefore, for this reason, it also had its information extracted. It is registered in TriTrypDB under ID LINF_220012800. It is located on chromosome 22, at location LinJ.22: 320,750.. 322,795 (-), of the species *L. infantum*, strain JPCM5. It has an isoelectric point of 3.77, a molecular weight of 63230kDa, and a length of 681. It has several epitopes registered in the IEDB and its information is described in Table 5.

Table 5 – A2 antigen epitopes (ID LINF_220012800) described in the IEDB

Epitope	Sequence	Location on Protein	Confidence
857710	PQSVGPLSVGPQSVGP	49-64	High
870835	MKIRSVRPLVVLLVC	1-15	Low
871486	VVLLVCVAAVLALSA	10-24	Low
871285	SVGPQAVGPLSVGPQ	100-114	Low
871410	VGPQAVGPLSVGPQS	101-115	Low
870994	PQAVGPLSVGPQSVG	103-117	Low
871025	QAVGPLSVGPQSVGP	104-118	Low
870796	LVCVAAVLALSASAE	13-27	Low
871286	SVGPQSVGPLSVGPL	147-161	Low
870996	PQSVGPLSVGPLSVG	150-164	Low
871406	VGPLSVGPLSVGPQS	153-167	Low
870781	LSVGPLSVGPQSVGP	156-170	Low
871380	VAAVLALSASAEPHK	16-30	Low

871432	VLALSASAEPHKAAV	19-33	Low
870773	LSASAEPHKAAVDVG	22-36	Low
834522	SAEPHKAAVDV	25-35	Low
871174	SAEPHKAAVDVGPLS	25-39	Low
870783	LSVGPQSVGPLSVGS	340-354	Low
871288	SVGPQSVGPLSVGSQ	341-355	Low
870360	GPQSVGPLSVGSQSV	343-357	Low
870999	PQSVGPLSVGSQSVG	344-358	Low
871284	SVGPLSVGSQSVGPL	346-360	Low
871409	VGPLSVGSQSVGPLS	347-361	Low
870984	PLSVGSQSVGPLSVG	349-363	Low
870784	LSVGSQSVGPLSVGP	350-364	Low
871413	VGSQSVGPLSVGPQS	352-366	Low
870375	GSQSVGPLSVGPQSV	353-367	Low
871157	RSVRPLVVLLVCVAA	4-18	Low
870045	DVGPLSVGPQSVGPL	41-55	Low
871408	VGPLSVGPQSVGPLS	42-56	Low
870356	GPLSVGPQSVGPLSV	43-57	Low
870980	PLSVG	44-48	Low
870983	PLSVGPQSVGPLSVG	44-58	Low
870782	LSVGPQSVGPLSVGP	45-59	Low
871287	SVGPQSVGPLSVGPQ	46-60	Low
871412	VGPQSVGPLSVGPQS	47-61	Low
870359	GPQSVGPLSVGPQSV	48-62	Low
870998	PQSVGPLSVGPQSVG	49-63	Low
871063	QSVGPLSVGPQSVGP	50-64	Low
871283	SVGPLSVGPQSVGPL	51-65	Low
871407	VGPLSVGPQSVDVSP	593-607	Low
871144	RPLVVLLVCVAAVLA	7-21	Low
871411	VGPQSVGPLSVGPQA	91-105	Low

870358	GPQSVGPLSVGPQAV	92-106	Low
871062	QSVGPLSVGPQAVGP	94-108	Low
871282	SVGPLSVGPQAVGPL	95-109	Low
870355	GPLSVGPQAVGPLSV	97-111	Low
870981	PLSVGPQAVGPLSVG	98-112	Low

4- Discussion

This systematic review aimed to identify, among the various antigens tested against Canine Visceral Leishmaniasis (CVL), those with better protection rates that could have their epitopes used in the construction of a new vaccine antigen. Of the antigens researched in the studies that meet the quality criteria of this review, 73.9% (34/46) are second-generation antigens and 26.1% (12/46) are third-generation antigens, mostly tested in the laboratory (43/45).

All these studies tested the immunogenicity of their antigens, with assessments of humoral and cellular immune responses. These parameters were considered essential for the studies to be analyzed in this review because although most studies that test vaccine antigens only evaluate the humoral immune response as a way of confirming immunogenicity when it comes to Canine Visceral Leishmaniasis, it is relevant that it be. The cellular immune response was also evaluated, since the presence of antibodies is not sufficient to combat the parasite (Loria-Cervera & Andrade-Narváez, 2014). This is because protozoa of the genus *Leishmania* are obligate intracellular and antibodies cannot neutralize them after entering cells, despite being important because they play classic mechanisms such as opsonization, activation of the complement system, and others that are significant in combating intracellular microorganisms. (Casadevall, 2018). For this reason, to fight them, there must be activation of cells such as Natural Killer and CD8+ T lymphocytes, responsible for eliminating infected cells, in addition to the polarization of the immune response to the Th1 type, with CD4+ T cells predominantly producing cytokines such as IL -2, IFN-γ and TNF-α and others that will cause macrophages to be classically activated and increase their phagocytosis capacity, with consequent destruction of the parasite more efficiently (Liu & Uzonna, 2012).

To confirm that the antigen tested stimulated the cellular immune reaction, 38.9% (7/18) of the studies used the ELISA test to identify and quantify the cytokines produced by cells of vaccinated animals, especially IFN-γ which, when in greater amounts characterize the Th1

profile and IL-10, a cytokine considered anti-inflammatory, related to the susceptibility of dogs to the parasite as it is characteristic of the Th2 response profile and which, therefore, must be in smaller amounts (Turchetti et al., 2015). With the same purpose, 27.8% (5/18) of the studies used RT- qPCR. To identify the predominant cell types, 33.3% (6/18) of the studies used the lymphoproliferation technique and 16.7% (3/18) used flow cytometry. Also tested in 11.1% (2/18), the ability of macrophages to destroy the parasite through the Canine macrophage Leishmanicidal Assay (CMLA) and 16.7% (3/18) of the articles also tested the production of Nitric Oxide (NO), an important molecule produced by monocytes to combat intracellular parasites such as *Leishmania* spp., by the direct action of IFN-γ from TCD4+Th1 (Olekhnovitch & Bousso, 2015). All these tests are important and should be used by studies that want to prove that their antigen stimulates the type of immune response identified by the literature as responsible for dog's resistance to CVL (Gonçalves et al., 2019).

In addition to the cellular immune response, it is also valid that the humoral immune response is analyzed. Antibodies, despite not having significant efficacy in combating the parasite, also contribute to the pathogenesis of the disease, by forming immune complexes that are deposited in joints, kidneys, ocular and vascular structures, causing clinical manifestations such as polyarthritis, renal failure, uveitis, and vasculitis (Ciamella & Corona, 2003). Antibodies must be evaluated, because, in addition to indicating the stimulus to the individual's humoral immune response against the antigen, they can also be used to differentiate infected from vaccinated animals, considering that immunoglobulins are specific and those that bind to the vaccine antigen with a structure unique, such as vaccine antigens constructed in the laboratory, using bioinformatics tools, will hardly bind to the antigens of the parasite that causes the disease.

To analyze the production of immunoglobulins against antigens in vaccinated dogs, ELISA tests were used in 100% (18/18) of the studies and IFAT in 5.6% (1/18) of the studies. The humoral immune response of two antigens was evaluated and presented clearly, in which the FML antigen (Borja-Cabrera et al., 2008) stimulated antibody production in 423 of 432 (98%) vaccinated dogs, representing 98 % and pMOK-Kmp11/-TRYP/-LACK/-GP63 antigen (Rodriguez-Cortés et al., 2007) stimulated immunoglobulin production in 100% (6/6) of vaccinated dogs. Both studies showed that specific immunoglobulins against these antigens were also produced by unvaccinated groups, 15.6% (98/588) in the study by Borja-Cabrera et al. (2008) and 33.33% (2/6) in the study by Rodriguez-Cortés et al., 2007. This would probably not occur if the antigens were of a unique genetic structure and not present in the wild parasite, which would be valid in populations with a large number of vaccinated dogs, where it would

be possible to identify the immunized and the truly infected, for a more reliable estimate of the prevalence of the disease in the evaluated place.

The ELISA test is considered the gold standard for the identification of animals infected with the protozoan, recommended by the Brazilian Ministry of Health as a confirmatory test for CVL (Silva et al., 2016). Therefore, it is common for studies on vaccine efficacy to use it as a way of identifying a humoral immune response against the antigen and to confirm the infection of the animals used in the experiment, which occurred in 34.05% (16/47) of the trials. In addition to ELISA, q-PCR tests (35/47); direct view (7/47); parasitic culture (21/47); Direct Agglutination (DAT) (2/47) and delayed hypersensitivity test (DTH) (2/47).

The results of these diagnostic methods, together with the evaluation of clinical signs, were used to calculate the efficacy rate against CVL and *L. infantum* infection. Among the antigens evaluated, only the LACK antigen, administered to animals in two different ways in different vaccinations (Ramiro et al., 2003) showed a protection rate of 80% (RR = 0.2, 95% CI: 0.03 – 0.66) against LVC and 60% (RR = 0.4, 95% CI: 0.11 – 0.93) protection against *L. infantum* infection. This antigen, therefore, was chosen among the others to have its characteristics searched in the TriTrypDB database. In addition to this, the A2 antigen was also selected, as it is relevant in the literature. It is important to highlight that the studies that did not evaluate clinical signs are extremely important, however, they were not applicable to the objectives of this review.

The LACK antigen (ID LINF_280034700) has an isoelectric point (PH value where the positive and negative charges of a molecule are equivalent) of 6.49. The isoelectric point, among other factors, determines the solubility of proteins and, therefore, must be considered in studies that aim to build new protein vaccine antigens. pH is different from the isoelectric point of the protein, preventing its precipitation (Nogueira, 2019). This antigen has a molecular weight of 34373kDa and a length of 312, important measures that must be considered, since the greater the molecular weight of a peptide, the greater the number of epitopes that compose it and the greater its immunogenicity, which is also influenced by the length. and by the complexity of the protein, both affected by its physicochemical composition and amino acid position (Mahanty et al., 2015). This information is also important when it comes to developing a recombinant vaccine from a protein antigen constructed using bioinformatics tools.

In the development of a recombinant immunogen, several techniques can be used, including the inclusion of the genetic sequence of the protein in a bacterial plasmid, so that it is initially cloned and incorporated into a bacterium responsible for recombinantly expressing the target peptide (Gibertoni et al., 2010). After expression, it is necessary that this protein is

purified and separated from all molecules from the bacterium that expressed it. Therefore, it is important to confirm its expression, which can be done by a process of gel electrophoresis (Nogueira, 2019), where it will be identified by its length and molecular weight (Gibertoni et al., 2010). This information is relevant for this review, however, data on the epitopes that make up this antigen would also be needed, which so far are not registered in the IEDB. This makes it difficult to determine these molecules for use in a new vaccine antigen. However, these data can be obtained by means of bioinformatics tools, indicated in the IEDB itself, something objectified by the authors of this review in the next phase of the study.

The A2 antigen, despite not showing significant protection rates according to the calculations made in this review, showed promise when researched in the literature and, for this reason, its information was also extracted. Its registration number in TriTrypDB is LINF_220012800 and it has an isoelectric point of 3.77, a molecular weight of 63230kDa, and a length of 681. It has 48 epitopes registered in the IEDB (Table 5), however, only one was classified as high confidence in this way, it must be considered for the construction of a new vaccine antigen. The epitope number 857710, sequence PQSVGPLSVGPQSVGP and location 49-64 in the protein, can have its information predicted in predictors indicated in the IEDB, to be framed in subsequent analysis. This antigen has been tested in several species and its described epitopes can also be tested for its protective capacity in dogs, in order to be incorporated into a new vaccine antigen.

In conclusion, this systematic review suggests that two antigens have the potential to be used in the construction of a new multiepitope vaccine. The LACK antigen has shown promise in different studies, demonstrably stimulating the humoral immune response, with significant rates of protection against disease and infection. However, it is necessary to better define the epitopes that compose it, through prediction tools, to determine those that best stimulate the protection of animals. Of the epitopes of the A2 antigen, already described in the IEDB, one considered to be of high confidence can be used in a new vaccine antigen. It is important to say that the purpose of this review is to find epitopes that can protect animals against infection and against the appearance of clinical signs of the disease, guaranteeing their quality of life, becoming an efficient measure of public health that corroborates with the well-being animal. Therefore, other antigens analyzed in this review, which demonstrated good stimulation of cellular and humoral immune responses, such as FML antigen responses, will also be evaluated to fulfill the same objective, with studies that manage to determine their protection rates.

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6- Supplementary tables

Supplementary Table S1 – Guidelines of PRISMA statement

Section/topic	#	Checklist item	Reported on §
		Title	
Title	1	Identify the report as a systematic review, meta-analysis, or both	§1
		Abstract	
Structured Summary	2	Provide s structured summary including, as applicable: background; objectives; data sources; study eligibility criteria; participants and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number	§1
		Introduction	
Rationale	3	Describe the rationale for the review in the context of what is already known	§1-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS)	§4
		Methods	
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	§1,2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	§2 Tab.[1] S2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	§1
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	§Tab. S2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	§3 Tab. S3
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	§5-6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	§4-6 Tab. S2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis	§6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	§7
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I2) for each meta-analysis.	§7
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	§6

Additional analysis	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	§7
Results			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	§1 Fig[2]. 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Tab. S4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	§1 Fig.2,3
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	§2-7 Tab. 1 – 5 Tab. S5-S6
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	-
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	-
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see item 16)]	-
Discussion			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers)	§1
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	§2 – 6
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	§7
Funding			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	§1

[1]Tab.: Table;[2]Fig.: Figure

Supplementary Table S2 – Search terms used in CABI, Cochrane, Pubmed, Scielo, Science Direct, Scopus and Web of Science databases, based on the PICOTS terms.

PICOTS	Search terms
Population	Animal* OR Human* OR Person OR People OR Patient
Intervention	Vaccin* AND Leishman* OR Epitope AND Leishman* OR Antigen AND Leishman*
Comparison	prophyla* OR prevalen* OR persisten* OR incidenc* OR epidemiol* OR control* OR prevent* OR efficacy OR effect* OR immun* OR protect* OR safe*
Outcomes	"parasit* load" OR "parasit* burden" OR "cell immune response" OR "humoral immune response" OR antibod* OR serolog* OR "clinical signs" OR cell* OR Cytokines
Time	-
Setting	Systematic review

Supplementary Table S3 – Inclusion and exclusion criteria for selection of articles in this systematic review.

Inclusion criteria	Exclusion criteria
• All countries • All years • Canine visceral • Studies on vaccines • *L. infantum* • Vaccines tested on	• *In vitro* and *In silico* studies • Cohort studies • Case-control studies • Cross-sectional studies • Case reports and Reviews • Diagnostic performance of tests • Therapeutics • Languages other than English, Spanish or Portuguese • Full-text not available • No information about control group • No information about vaccine antigen, dose, route, number • No challenge performed • No information about challenge dose, route, strain or time • No information about evaluation of humoral immune • No information about evaluation of cellular immune

- No information about parasite load
- No information about vaccine protection rate
- Study with whole parasite
- Study with excreted and secreted antigens

Supplementary Table S4 - Studies not selected by quality criteria in this review

First author, year	Reason
Aguiar-Soares, 2020	No challenge
Araújo, 2011	No challenge
Araújo, 2009	No challenge
Araújo, 2008	No challenge
Bongiorno, 2013	Cellular immune response not evaluated
Borja-Cabrera, 2002	Humoral immune response not evaluated
Carrillo, 2008	No challenge
Carson, 2009	No challenge
Costa-Pereira, 2015	Cellular immune response not evaluated
Cotrina, 2018	Humoral immune response not evaluated
Silva, 2011	Cellular immune response not evaluated
Lima, 2010	Cellular immune response not evaluated
Fernandes, 2014	Humoral immune response not evaluated
Fernández Cotrina, 2018	Humoral immune response not evaluated
Fujiwara, 2005	Humoral immune response not evaluated
Gradoni, 2005	Humoral immune response not evaluated
Grimaldi, 2017	Humoral immune response not evaluated
Holzmuller, 2005	Cellular immune response not evaluated
Lima, 2010	Cellular immune response not evaluated
Marcondes, 2011	Humoral immune response not evaluated
Martinez-Rodrigo, 2019	No challenge
Mohammadi-Ghalehbin, 2017	No challenge
Molano, 2003	Humoral immune response not evaluated
Montalvo-Alvarez, 2008,	Humoral immune response not evaluated
Montoya, 2021	Humoral immune response not evaluated
Moreira, 2016	No challenge
Moreno, 2012	No challenge
Moreno, 2014	No challenge

Nogueira, 2005	Humoral immune response not evaluated
Ogunkolade, 1988	Humoral immune response not evaluated
Oliva, 2014	Humoral immune response not evaluated
Parra, 2007	Cellular immune response not evaluated
Pinheiro, 2018	Humoral immune response not evaluated
Regina-Silva, 2016	Humoral immune response not evaluated
Resende, 2016	Cellular immune response not evaluated
Resende, 2013	Cellular immune response not evaluated
Saldarriaga, 2006	No challenge
Saraiva, 2006	Humoral immune response not evaluated
Testasicca, 2014	Humoral immune response not evaluated
Toepp, 2018	Cellular immune response not evaluated
Velez, 2020	Humoral immune response not evaluated

Supplementary Table S5 – Detailed information about the vaccinations in the trials that performed this analysis among those selected by this systematic review.

		First vaccination		Second vaccination			Third vaccination			Fourth vaccination		
First author,year	**Route**	**Antigen**	**Dose**	**Antigen**	**Interval[a]**	**Dose**	**Antigen**	**Interval[a]**	**Dose**	**Antigen**	**Interval[a]**	**Dose**
Abeijon, 2016	SC[b]	Li-ntf2	50µg	Same[j]	30 d	Same[j]	Same[j]	30 d	Same	NP[k]	NP[k]	NP[k]
Alcolea, 2019	IN[c]	pPAL-LACK + pPAL-canIL12-p35 + pPALcanIL12-p40	200µg[h]+ 20µg[h] + 20µg[h]	Same[j]	15 d[j]	Same[j]	Same[j]	15 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Borja-Cabrera, 2009	IM[f]	VR1012-NH36	750 µg	Same[j]	21 d[j]	Same[j]	Same[j]	21 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Borja-Cabrera, 2008	SC[b]	FML	1mL[i]	Same[j]	21 d	Same[j]	Same[j]	21 d	NP[k]	NP[k]	NP[k]	NP[k]
Carcelen, 2009	SC[b]	Q protein	100 µg	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Carcelen, 2009	SC[b]	Q protein	100 µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
De Lima, 2010	SC[b]	FML	1 mL[i]	Same[j]	21 d[j]	Same[j]	Same[j]	21 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
De Lima, 2010	SC[b]	FML	1mL[i]	Same[j]	21 d[j]	Same[j]	Same[j]	21 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Fernandes, 2008	SC[b]	rA2	100 µg	Same[j]	21 d[j]	Same[j]	Same[j]	21 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Fernandes, 2008	SC[b]	rA2	100 µg	Same[j]	21 d[j]	Same[j]	Same[j]	21 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Fernandes, 2008	SC[b]	rA2	100 µg	Same[j]	21 d[j]	Same[j]	Same[j]	21 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Fernandes, 2008	SC[b]	rA2	100 µg	Same[j]	21 d[j]	Same[j]	Same[j]	21 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	HASPB1	100 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	HASPB1	100 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	HASPB1	100 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	H1	100 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	H1	100 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	H1	100 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	HASPB1 + H1	100 µg[h] + 100 µg[h]	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	HASPB1 + H1	100 µg[h] + 100 µg[h]	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]

Goto, 2007	ID[g]	HASPB1 + H1	100 µg[h] + 100 µg[h]	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	MML	45 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	MML	45 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Goto, 2007	ID[g]	MML	45 µg	Same[j]	30 d	45 µg	Same[j]	30 d	45 µg	NP[k]	NP[k]	NP[k]
Petitdidier, 2016	SC[b]	LaPSA-38S	25 µg	Same[j]	28 d[j]	Same[j]	Same[j]	28 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Petitdidier, 2016	SC[b]	LaPSA-12S	25 µg	Same[j]	28 d[j]	Same[j]	Same[j]	28 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Petitdidier, 2019	SC[b]	A17G + A17E + E34PC	25 µg[h] + 25 µg[h] + 10 µg[h]	Same[j]	28 d[j]	Same[j]	Same[j]	28 d[j]	Same[y]	NP[k]	NP[k]	NP[k]
Poot, 2009	SC[b]	rJPCM5_Q[d]	70µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Poot, 2009	SC[b]	rJPCM5_Q[d]	70µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Poot, 2009	SC[b]	rJPCM5_Q[d]	70µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Poot, 2009	SC[b]	rJPCM5_Q[e]	70µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Poot, 2009	SC[b]	rJPCM5_Q[e]	70µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Poot, 2009	SC[b]	rJPCM5_Q[e]	70µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Poot, 2006	SC[b]	rCPA + rCPB	50 µg[h] + 50 µg[h]	Same[j]	28 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Poot, 2006	SC[b]	rCPA + rCPB	50 µg[h] + 50 µg[h]	Same[j]	28 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Ramiro, 2003	SC[b]	DNA-LACK	100µg	Same[j]	15 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Ramiro, 2003	SC[b]	DNA-LACK	100µg	rVV-LACK	15 d[j]	10^8 PFU	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Ramos, 2008	SC[b]	DNA-LACK	100 µg	rVV – LACK	15 d	10^7 PFU	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Ramos, 2008	SC[b]	DNA-LACK	100 µg	MVA-LACK	15 d	10^8 PFU	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]

Ramos, 2009	SC[b]	pORT-LACK	100 µg	MVA-LACK	15 d	10^8 PFU	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Rodriguez-Cortés, 2007	ID[g]	pMOK-Kmp11/-TRYP/-LACK/-GP63	200µg[w]	Same[j]	15 d[j]	Same[j]	Same[j]	21 d[j]	Same[j]	Same[j]	Same[j]	Same[j]
Shahbazi, 2015	SC[b]	pcDNA-A2-CPACPB−CTEGF P (cSLN)	200µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
Shahbazi, 2015	SC[b]	pcDNA-A2-CPACPB−CTEGFP (Electroporation)	200µg	Same[j]	21 d[j]	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
M. Shahbazi, 2015	SC[b]	*L. tarentolae* A2-CPA-CPB-CTE EGFP	2x10^7	Same[j]	21 d	Same[j]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]	NP[k]
R. Sima, 2005	IM[f]	pCB6-cpa + pCB6-cpb	100µg[h] + 100µg[h]	Same[j]	30 d	Same[j]	rCPA + rCPB + ODN cpg	30 d	200µg +200µg + 230 µg	NP[k]	NP[k]	NP[k]

[a]Interval: interval between the vaccinations; [b]SC: Subcutaneous; [c]IN: Intranasal; [d]rJPCM5_Q: Antigen produced by *E. coli;* [e]rJPCM5_Q: Antigen produced by *Baculovirus;* [f]IM: Intramuscular; [g]ID: Intradermal; [h]Dose of each antigen; [i]Dose recommended by the vaccine manufacturer; [j]Same: Same antigen or dose as first dose; [k]NP: Not performed

Supplementary Table S6 – Detailed data on the humoral immune response evaluated through antibodies against the vaccine antigen, produced after vaccination, in trails that performed this analysis among those selected by this systematic review.

First author, year	Antigen/Adjuvant	ELISA (Antibodies)[a]		Cut off (ELISA)
		NVac[c] (%)	NC[d] (%)	
Abeijon, 2016	Li-ntf2			
Alcolea, 2019	pPAL-LACK	NP[e]	NP[e]	NP[e]
Borja-Cabrera, 2009	VR1012-NH36	UE[f]	UE[f]	UE[f]
Borja-Cabrera, 2008	FML	423/432 (98)	92/588 (15.6)	Abs. 492 nm = 450
Carcelen, 2009	Q-Protein	UE[f]	UE[f]	UE[f]
Carcelen, 2009	Q-Protein + Q-Protein	UE[f]	UE[f]	UE[f]
Fernandes, 2008	rA2/Saponin	UE[f]	UE[f]	UE[f]
Goto, 2007	HASPB1	UE[f]	UE[fg]	UE[f]
Goto, 2007	H1	UE[f]	UE[fg]	UE[f]
Goto, 2007	HASPB1 + H1	UE[f]	UE[fi]	UE[f]
Goto, 2007	MML	UE[f]	UE[fh]	UE[f]

Petitdidier, 2016	LaPSA-38S/QA-21	UE[f]	UE[f]	UE[f]
Petitdidier, 2016	LaPSA-12S/QA-21	UE[f]	UE[f]	UE[f]
Petitdidier, 2019	A17G + A17E + E34PC/QA-21	UE[f]	UE[f]	UE[f]
Poot, 2009	rJPCM5_Q/MDP	UE[f]	UE[f]	UE[f]
Poot, 2009	rJPCM5_Q/ Aluminum hydroxide	UE[f]	UE[f]	UE[f]
Poot, 2009	rJPCM5_Q/ ISCOMatrix C	UE[f]	UE[f]	UE[f]
Poot, 2009	rJPCM5_Q/MDP	UE[f]	UE[f]	UE[f]
Poot, 2006	rCPA + rCPB	UE[f]	UE[f]	UE[f]
Ramiro, 2003	DNA-LACK	UE[f]	UE[f]	UE[f]
Ramiro, 2003	DNA-LACK + rVV-LACK	UE[f]	UE[f]	UE[f]

Rodriguez-Cortees, 2007	pMOK-Kmp11/-TRYP/-LACK/-GP63	6/6 (100)	2/6 (33.33)	9 EU
Shahbazi, 2015	pcDNA-A2-CPACPB−CTEGFP (cSLN)	UE[f]	UE[f]	UE[f]
Shahbazi, 2015	pcDNA-A2-CPACPB−CTEGFP (Electroporation)	UE[f]	UE[f]	UE[f]
M. Shahbazi, 2015	*L. tarentolae* A2-CPA-CPB-CTE EGFP	UE[f]	UE[f]	UE[f]
M. Shahbazi, 2015	*L. tarentolae* A2-CPA-CPB-CTE EGFP	UE[f]	UE[f]	UE[f]
R. Sima, 2005	pCB6-cpa + pCB6-cpb; rCPs + ODN CPG	UE[f]	UE[f]	UE[f]
R. Sima, 2005	pCB6-cpa + pCB6-cpb; rCPs + ODN CPG	UE[f]	UE[f]	UE[f]

[a]Number of animals that developed a humoral immune response against the vaccine antigen confirmed by ELISA; [b]Number of animals that developed a humoral immune response against the vaccine antigen confirmed by IFAT; [c]NVac: Vaccinated group; [d]NC: Control group; [e]NP: Not performed; [f]UE: Unable exctract; [g]Control: Ajduvant Montanide; [h]Control: Adjuvant MPL-SE; [i]Control: Placebo